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Prognostic aspects of hepatocellular carcinoma

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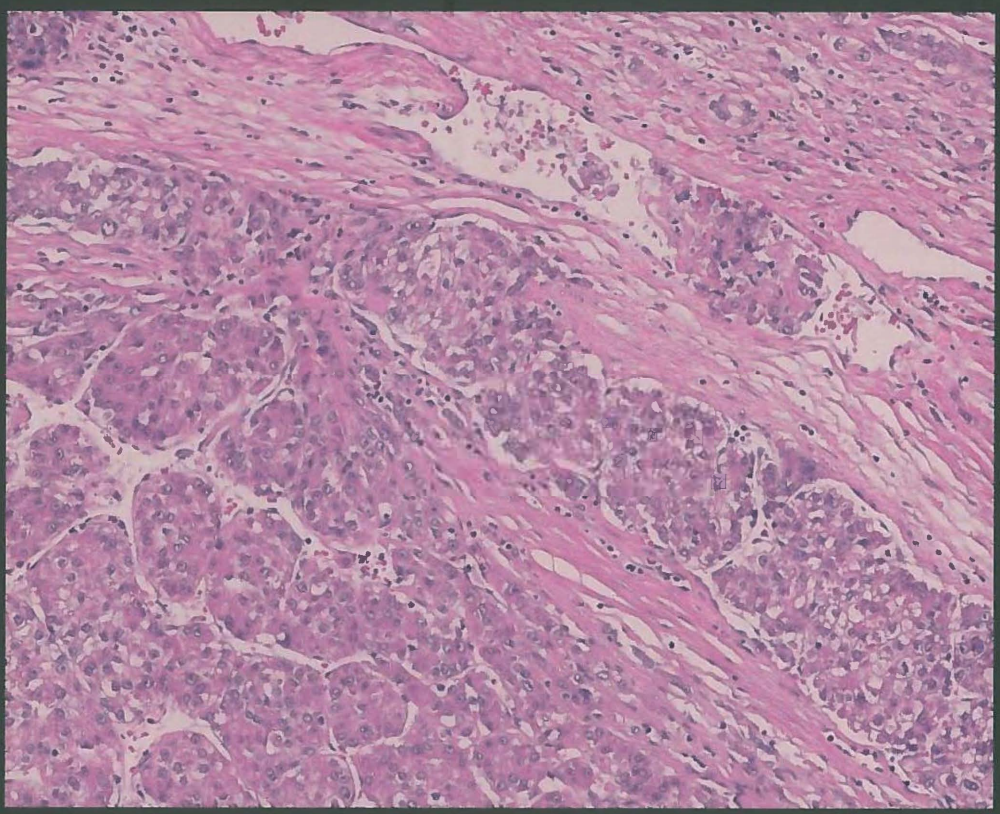
Prognostic aspects of Hepatocellular Carcinoma



Hironori Kusano

Prognostic aspects of Hepatocellular Carcinoma

Hironori Kusano

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Stellingen behorende bij het proefschrift

Prognostic aspects of Hepatocellular Carcinoma

Hironori Kusano, December 4, 2013

1. Microvascular invasion in hepatocellular carcinoma is not only influenced by tumor characteristics but also by changes in the adjacent non-cancerous tissue. (This thesis)

2. The increased expression of placental growth factor and vascular endothelial growth factor receptor-1 in the tissue adjacent to hepatocellular carcinoma can stimulate the generation of abnormal vessels that are permissive to invasion. (This thesis)

3. The microvessels evaluated in microvessel density scoring do not represent the microvessels involved in microvascular invasion. (This thesis)

4. The association of microvascular invasion in hepatocellular carcinoma with the disappearance of Epithelial-Cell-Adhesion-Molecule positive ductules signifies the involvement of epithelial-mesenchymal transition (This thesis).

5. Combination therapy of interferon-α with sorafenib may improve the outcome of sorafenib monotherapy in a selected group of patients with hepatocellular carcinoma. (This thesis)

6. Microvessel density in hepatocellular carcinoma is not an appropriate parameter to evaluate the efficacy of anti-angiogenic drugs. (This thesis)

7. Pegylated interferon has a stronger antitumor effect than non-pegylated interferon. (This thesis)

8. *I no naka no kawazu, taikai wo shirazu.* (A frog in a well does not know the great sea). (Japanese proverb)

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Contents

Abbreviations and acronyms

AFP	α-fetoprotein
AUC	area under curve
BCLC	Barcelona Clinic Liver Cancer
CK	cytokeratin
CLIP	Cancer of the Italian Group
CSC	cancer stem cell
CT	computed tomography
DCP	des-γ-carboxy prothrombin
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
FGF	fibroblast growth factor
Gd-EOB-MRI	gadoxetate disodium-enhanced magnetic resonance imaging
HAI	hepatic intraarterial infusion
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
JIS	Japan Integrated Staging
K	lysine
Mab	monoclonal antibody
macro-VI	macrovascular invasion
micro-VI	microvascular invasion
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MVD	microvessel density
OLT	orthotopic liver transplantation
PEG-IFN	pegylated interferon
PIVKA-II	protein induced by vitamin K absence or antagonist II
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PIGF	placental growth factor
PON1	paraoxonase 1
RCT	randomized controlled trial
RFA	radiofrequency ablation
SIN1	stress-activated protein kinase interacting protein 1
SIRT2	sirtuin 2
TACE	transarterial chemoembolization
TGF	transforming growth factor
TKI	tyrosine kinase inhibitor
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
5-FU	5-fluorouracil

Chapter 1

Introduction and Integration of Main Results

General Introduction

Primary liver cancer, of which hepatocellular carcinoma (HCC) represents the major subtype accounting for 85% to 90%, is the sixth most common cancer globally, and the third most common cause of cancer-related death [Ferlay et al, 2010]. HCC usually occurs in cirrhotic livers following longstanding chronic inflammation of the liver regardless of its etiology. Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection, alcohol related liver disease, obesity and some metabolic disorders such as diabetes are major risk factors for chronic liver disease, subsequent cirrhosis and potential development of HCC. There are huge geographic variations in the incidence rates of HCC. The areas of high incidence are eastern and south-eastern Asia and Africa because Hepatitis B and/or Hepatitis C are endemic in these areas [El-Serag, 2011]. In Western countries the incidence rate of HCC is lower, yet increasing because of other risk factors, such as obesity and type II diabetes mellitus.

Cancer treatment strategy is usually based on the Tumor Nodes Metastases staging system which incorporates the size and extent of the primary tumor, the status of regional lymph nodes, and the existence of distant metastasis. For HCC, liver disease stage, such as the Child-Pugh classification is also important to determine the treatment strategy and to predict prognosis. Current staging systems for HCC include both tumor and liver disease stage. The Cancer of the Liver Italian Group (CLIP) scoring system incorporates tumor morphology, the existence of portal vein thrombosis, the value of α-fetoprotein (AFP), and Child-Pugh stage [The CLIP investigators, 1998] and has been well validated world wide as a staging system predictive of prognosis. The Japan Integrated Staging (JIS) score combines Child-Pugh score and TNM stage based on the Liver Cancer Study Group of Japan [Kudo et al., 2003], and a new system incorporating the JIS score and the value of serum tumor markers: AFP, AFP-L3, Des-γ-carboxy prothrombin (DCP), has recently been proposed [Kitai et al., 2008]. These two proposed scoring systems from Japan were better prognostic systems than the CLIP score among the Japanese patient groups [Kudo et al., 2004; Kitai et al., 2008], yet further external validation is required [Marrero et al., 2010]. The Barcelona Clinic Liver Cancer (BCLC) staging system is much more intended to determine a therapeutic option for individual patients with HCC, rather than to predict prognosis [Llovet et al., 1999]. The widely applied BCLC treatment strategy includes tumor stage, performance status and liver function, and links the stage of the disease to a specific treatment option. Briefly, very early or early stage of the disease is linked to the curative treatment options consisting of surgical resection, liver transplantation or local ablation. The intermediate stage to transarterial chemoembolization (TACE) and the advanced stage to sorafenib therapy which are both palliative treatments while the

terminal stage can only be addressed by symptomatic treatment [Bruix and Sherman, 2011].

An important factor that contributes to the limitations of therapeutic management of HCC is the fact that by far the majority of HCC develops in patients with cirrhosis. Liver cirrhosis represents longstanding chronic liver disease with serious clinical complications, such as portal hypertension and progressive loss of specific liver functions. Hence, the therapeutic intervention of HCC requires a combined approach of aggressively dealing with a malignant tumor and careful consideration of a diseased organ with limited capacity of regeneration in a sick patient.

Liver transplantation (LT) is regarded as a curative treatment for HCC which is associated with the lowest risk of tumor recurrence. Selection of HCC patients for LT is usually based on the Milan criteria [Mazzaferro et al., 1996] which states that patients with solitary HCC of 5cm or less in diameter, or 2 or 3 HCC nodules of which each tumor is 3cm or less in diameter, are considered eligible for LT. The patients within these criteria have an expected 5-year overall survival rate of 70% with recurrence less than 10% after LT, which is similar to the 5-year survival rate of non-HCC individuals [Colombo et al., 2013]. Since organs available for LT are so scarce and the number of patients requiring LT far exceeds the number of organs available, these criteria can not be rigidly applied. Surveys on extending the criteria seem to allow limited adjustment. The Metroticket investigator study group carried out the analysis of patients with HCC beyond the Milan criteria who underwent LT, which was a retrospective, and so far the largest study [Mazzaferro et al., 2009]. Through their study, they set up a prognostic model based on the size of the largest tumor, number of tumors and microvascular invasion (micro-VI), all of which are regarded as independent prognostic factors, and found that patients with micro-VI negative HCC beyond the Milan criteria but within the so called "Up-to-Seven" criteria, where the seven refers to the sum of the size (cm) of the largest tumor and number of tumors, have a similar acceptable 5-year survival to those with micro-VI positive HCC within the Milan criteria. This finding suggests that this particular patient group beyond the Milan criteria might be eligible for LT. However, there is no tool predictive of micro-VI before transplantation, and therefore, it is not recommended that indication of LT should rely on micro-VI [Clavien et al., 2012]. Besides the dilemma of micro-VI, their study also highlighted the difficulties of radiological assessment of size and number of tumors, as documented repeatedly [Sotiropoulos et al., 2005 & Taouli and Krinsky, 2006]. Substantial numbers of patients classified as being beyond the Milan criteria preoperatively (444 patients, 28.5%) fell within these criteria after pathological evaluation, and had an excellent survival similar to those within the Milan criteria [Mazzaferro et al., 2009].

Both surgical resection and radiofrequency ablation (RFA) are potentially curative, and show excellent short-term outcome among patients with early or very early stage HCC. The former is eligible for non-cirrhotic patients and the latter for cirrhotic patients or those who have liver dysfunction. According to a recent report, no significant difference in 5-year survival rate is observed between these two therapies after adjusting covariates among patients with very early or early stage HCC [Wang et al, 2012]. There is another report that showed significant difference in overall survival between these two therapies [Karabulut et al., 2012]. In addition, since these therapies have no effect on underlying chronic liver disease, the 5-year disease free survival is as low as 50.8% in the resection group and 14.1% in the ablation group with surgical resection being superior to RFA [Wang et al., 2012].

For a long period, there was no standard treatment for patients with unresectable HCC. TACE showed a survival benefit among patients who have multiple HCCs without extrahepatic metastasis or vascular invasion and preserved liver function, and became a standard treatment for patients with intermediate stage HCC [Llovet and Bruix, 2003; Raoul et al., 2011]. It has recently been shown that TACE using drug eluting beads can improve patients' survival [Burrel et al., 2012]. TACE is used for patients with earlier stage HCC as well, who are not eligible for local ablation therapy due to tumor location [Colombo et al, 2013], and can also be used for patients on the waiting list for LT as a bridging therapy [Clavien et al., 2012]. An oral multikinase inhibitor, sorafenib, is the first molecular targeted drug which has been approved for advanced HCC in 2007 by the US Food and Drug Administration. The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial revealed efficacy of sorafenib in the treatment of HCC, i.e., both median survival and time to progression showed 3-month improvements by sorafenib therapy [Llovet et al., 2008]. Following the clinical success of sorafenib in the treatment for advanced HCC, research efforts are now performed on combination therapy of other drugs or surgical treatments with sorafenib.

In summary, HCC has become a global threat due to an increasing global incidence with relatively limited therapeutic options since curative treatment is only adequate for early stages and only a few therapeutic choices are available for non-surgically treatable patients. Furthermore there are no reliable markers to assess prognostic factors such as micro-VI before treatment.

Aims of the thesis

This thesis addresses two issues related to the prognosis of HCC, micro-VI and the sensitivity of HCC to interferon-α (IFN-α). The first part consists of studies on the pathogenetic background of micro-VI and in the second part the effects of IFN-α therapy are evaluated on the proliferative capacity of the tumor. Although micro-VI

is recognized as a vital prognostic factor, its pathogenesis is not well understood. In **chapter 2** the available methods to predict micro-VI before surgery are reviewed. The studies in **chapter 3 and 4** are designed to gain insight in the pathways that are involved in micro-VI and to establish a possible profile of tumors containing or lacking micro-VI. The studies are tissue based and were executed on archival tumor tissue of patients who underwent LT **(chapters 3 & 4**). HCC is a heterogenous cancer due to its various etiologies, hence its sensitivity to several types of anti-cancer drugs is variable. Based on the sorafenib outcomes we studied whether additional effects of combination therapy with IFN-α/sorafenib could be observed. In **chapters 5 & 6** we studied the effects of IFN-α administration with and without combination with sorafenib on several HCC-cell lines. The effects of both types of therapy were investigated by measuring the proliferative capacity of the tumor cells followed by subsequent *in vivo* experiments in nude mice.

Part I. Microvascular Invasion in HCC

The significance of microvascular invasion

Vascular invasion is defined as the presence of tumor cells within the vascular space in peritumoral tissue, and in HCC it commonly involves the local branches of portal and/or hepatic veins. In general, vascular invasion can be classified as macrovascular invasion (macro-VI) and micro-VI. Macro-VI is grossly recognizable and therefore detectable by conventional imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography. It is well known that macro-VI is a predictor for poor prognosis in patients with HCC [Llovet et al., 1999]. Due to a poor prognosis the preoperative diagnosis of macro-VI as determined by the above-mentioned imaging techniques is a contraindication to treat HCC with LT, surgical resection, local ablation therapy, or TACE [Bruix et al., 2011; EASL-EORTC, 2012]. According to the BCLC staging, HCC with macro-VI is classified into the advanced stage of the disease linking to the recommendation of sorafenib therapy, provided that the patient's performance status and liver function are preserved [Bruix et al., 2011; EASL-EORTC, 2012]. Among patients with the sorafenib treatment median overall survival is about 11 months [Llovet et al, 2008].

Similarly, micro-VI has also been shown to negatively influence the prognosis of HCC either after LT or resection. Rodríguez-Perálvarez et al reported in their review paper by using meta-analysis that the presence of micro-VI shortens the disease free- and overall survival after LT and disease free survival after surgical resection [Rodríguez-Perálvarez et al., 2013]. Overall survival and recurrence rate after surgical resection was clearly stratified when the extent of vascular invasion was categorized into no vascular invasion; micro-VI or macro-VI [Roayaie et al., 2009]. Another notable finding about the influence of micro-VI, among patients who underwent LT for HCC, 3- and 5-year survival rates were almost the same between patients with micro-VI positive HCC within the Milan criteria and patients with micro-VI negative HCC beyond the Milan criteria but within the “Up-to-seven criteria” [Mazzaferro et al., 2009]. The 5-year survival rate was more than 70% in both groups which is regarded as acceptable performance after LT for HCC. This suggests that patients with micro-VI negative HCC beyond the Milan criteria but within the "Up-to-seven criteria" could have been candidates for LT if micro-VI was detectable preoperatively, who are now excluded from a waiting list for LT due to being beyond the Milan criteria.

A major drawback of micro-VI is that in contrast to macro-VI, identification of micro-VI can only be done by histopathologic examination. Hence, by definition its diagnosis depends on the availability of tumor tissue. In practice, micro-VI can only be reliably diagnosed after surgical resection of the tumor. On a biopsy, evaluation of micro-VI may easily lead to sampling error. Another serious disadvantage of

micro-VI is the absence of widely recognized markers to predict the presence of micro-VI before surgery. This is a major obstacle to predict the tumor behavior and subsequent tailoring of the therapy of choice for HCC patients.

In **chapter 2** the available methods of predicting micro-VI before surgery is reviewed [Gouw et al., 2011]. Firstly, we investigated the relationship between micro-VI and tumor characteristics. Many studies showed a significant correlation between micro-VI and size and number of tumor. However it is to be noted that the size and number of tumor were evaluated pathologically after surgery, not radiologically before surgery. Since it is not easy to evaluate the size and numbers of tumor accurately in a cirrhotic liver, there is always some degree of discrepancy between radiological and pathological evaluation and this discrepancy is an absolute limitation of patient selection. Although several papers from Japan showed a significant correlation between micro-VI and gross classification proposed by the Liver Cancer Study Group of Japan, the same limitation exists. On the other hand, the advantage of radiological examination compared with the pathological one is that it is possible to evaluate the hemodynamics of the tumor. Witjes et al reported that the presence of washout demonstrated on dynamic contrast enhanced MRI was significantly associated with histological grade and micro-VI which are closely correlated with each other [Witjes et al., 2012]. Kim et al showed that peritumoral hypointensity seen on the hepatobiliary phase of gadoxetate disodium-enhanced MRI (Gd-EOB-MRI) is predictive of micro-VI [Kim et al., 2012]. Gadoxetate disodium is a recently-introduced hepatocyte specific contrast agent which allows to evaluate hemodynamics and hepatocyte's function of focal liver lesion at once. So far, Gd-EOB-MRI has become an indispensable tool in clinical practice for HCC. Further study regarding identification of micro-VI on Gd-EOB-MRI is needed.

Serum biochemical markers were also studied as a possible predictor of micro-VI before surgery. In some studies, a significant correlation was shown between micro-VI and the increase or rapid progression of AFP which is a conventional tumor marker for HCC [Pawlik et al., 2005; Vibert et al., 2010]. However AFP can be high with other liver diseases, so it is used in conjunction with AFP-L3 which is more HCC-specific. DCP, also known as protein induced by vitamin K absence or antagonist II (PIVKA-II), is another representative tumor marker for HCC, and has been shown to be associated with micro-VI [Kaibori et al., 2010]. There is no correlation between the serum levels of AFP and DCP in patients with HCC, suggesting that each tumor marker is reflecting a different tumor characteristic. It is considered that AFP is associated with tumor differentiation and DCP with metabolic dysfunction of tumor hepatocytes, but the mechanism of elevation of these markers are not fully understood. Murata et al showed that the increased expression of DCP was induced by hypoxia on HCC cells [Murata et al., 2010]. Moreover it was recently shown that the elevation of DCP is seen in patients

with HCC after sorafenib therapy [Ueshima et al., 2011]. These two recent findings suggest that the elevation of DCP may predict the presence of micro-VI, possibly due to hypoxia induced by microthrombus of portal vein. Further study is needed to clarify the mechanisms of elevation of these markers.

Other than AFP and DCP, paraoxonase 1 (PON1) has recently been introduced by Huang et al as a promising serum marker for micro-VI in HCC, which was identified by proteomic analysis [Huang et al., 2013]. Mínguez et al has shown that a 35-gene signature of vascular invasion identified by genome-wide gene-expression profiling improve the diagnostic accuracy for micro-VI of tumor size. [Mínguez et al., 2011]. More recently, Poté et al have found N-term acetylated histone H4 dimethylated at lysine (K)20, and acetylated at K16 were strongly expressed in HCC with micro-VI, which was identified using imaging mass spectrometry analysis [Poté et al., 2013]. Thus, not only genetic alterations but also epigenetic modification play a role in micro-VI. Various approaches are thus required to identify a new marker for micro-VI.

Possible factors related to the pathogenesis of micro-VI in HCC

In the studies on the molecular background of micro-VI in this thesis, tumor cells characteristics are studied but non-lesional liver parts are also included because by far the majority of HCC develops in a cirrhotic liver. This is in contrast with most other solid tumors which grow in an otherwise non-diseased organ as has been alluded to before.

A cirrhotic liver is the result of long term chronic liver disease, usually associated with years of chronic inflammation due to variable causes and invariably involving longstanding cellular injury alternating with regeneration activity. Without effective clearance of the injurious cause fibrotic scarring will develop which ultimately can lead to cirrhosis. Cirrhosis is not a passive end-stage scar formation but represents a dynamic pathologic process characterized by progressive fibrosis due to excessive abnormal deposition of extracellular matrix, deformation of the lobular architecture and disruption of the normal vascular architecture following parenchymal cell death and vascular remodeling [Crawford and Burt, 2012]. This is the background liver in which HCC usually develops.

One of the hallmarks of cancer is the ability of cancer cells to activate invasion and metastasis [Hanahan and Weinberg, 2011]. Vascular invasion is a complex process involving proliferation at the primary site, local invasion of tumor cells into adjacent tissue and intravasation of tumor cells. This process is part of the invasion-metastasis cascade in which vascular invasion is followed by extravasation, proliferation and colonization in the new site [Fidler, 2003; Talmadge and Fidler, 2010]. This multistep process involves an interplay between cancer cells and its microenvironment which are involved in reciprocal signaling [Hanahan and

Weinberg, 2011]. Therefore, the process of vascular invasion can be studied from the perspective of the tumor but also from its microenvironment. With regard to HCC, the microenvironment has undergone several alterations due to processes involved in the development of cirrhosis. Two of such vital processes are angiogenesis and epithelial-mesenchymal transition (EMT).

Angiogenesis and tumor vascularity: the relation with vascular invasion

Angiogenesis and tumor vascularity are 2 different but closely associated entities. Tumor vascularity reflects the pattern of blood vessels in and around the tumor whereas angiogenesis relates to the process of blood vessel formation.

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones. Since cancers need blood supply for oxygen and nutrients to grow, cancer cells and cancer surrounding cells release various growth factors such as vascular endothelial growth factor (VEGF) to stimulate angiogenesis [Folkman, 2002]. Such a pathological angiogenesis leads to changes of tumor vascularity. Normal liver receives a blood supply approximately 75% from the portal vein and the rest from the hepatic artery. In contrast, classical advanced HCCs receive their blood supply exclusively from abnormal arteries which lack the normal structure of portal tracts and accompanying veins and bile ducts. Therefore these tumor arteries are referred to as unpaired arteries. This is the reason why classical advanced HCCs are characterized by early enhancement in the arterial phase with washout in the portal phase during dynamic contrast-enhanced CT scan. On the other hand, early HCCs which contain portal tracts within the tumors do not only receive arterial blood flow from the unpaired arteries but also from pre-existing arteries and portal blood from the portal veins located within the intratumoral portal tracts [Nakashima et al., 1999; Kojiro, 2006].

So far, numerous studies have evaluated angiogenesis in HCCs [Fernández et al., 2009; Sanz-Cameno et al., 2010; Zhu et al., 2011]. One of the most frequently chosen approaches is to measure the gene and/or protein expression of angiogenic growth factors and their receptors. Most studies indicate the existence of a correlation between tumor progression and increased angiogenic factors. Another common histological approach to evaluate the angiogenic status of HCCs is to measure the microvessel density (MVD) within the tumors. There is substantial evidence that supports the notion that MVD correlates with the expression of angiogenic growth factors and prognosis in some cancers [Uzzan et al., 2004; Des Guetz et al., 2006; Rubatt et al., 2009; Iordache et al., 2010]. In HCC, MVD is usually measured by counting CD34-positive sinusoidal endothelial cells within tumors [Tanigawa et al., 1997]. Since tumor sinusoids are morphologically heterogeneous, definitions of MVD in HCC may vary. The variable definitions of MVD and the lack of standardized methods to count MVD are limitations of using

MVD as a measurement of angiogenesis. Nevertheless, within the mentioned limitations of MVD scoring as a measurement of angiogenic activity, MVD scoring has been applied in many studies that mainly focused on prognosis [Uzzan et al., 2004; Des Guetz et al., 2006; Rubatt et al., 2009; Iordache, 2010]. The majority of studies reports that high MVD is associated with poor prognosis after tumor resection or LT, although a few studies, including a previous study of our group, found a reverse correlation of high MVD with poor survival [Zeng et al., 2010].

One of the predictive independent factors for poor prognosis is micro-VI, but no association has been reported between MVD and micro-VI [Zeng et al., 2010]. The results of **chapter 3** are in line with these findings. We evaluated MVD according to a published method by others to enable comparisons with other publications and found no differences in MVD scores between HCCs with micro-VI and those without. Although a pathogenetic explanation for this lack of association between MVD and micro-VI is not available, it is conceivable that spatial differences of the microvessels involved in the 2 processes play a role. The microvessels evaluated in MVD scoring do not represent the microvessels involved in micro-VI. Histopathologically the latter is usually found in the vessels in the peritumoral stromal areas whereas MVD counts are predominantly performed in vascular hot spots at the peripheries of tumor nodules within the tumor boundaries.

Pathological angiogenesis also takes place in the non-cancerous background liver as part of vascular remodeling in the process of liver fibrosis. Firstly, as a general process during liver wound healing, repetitive liver damage induces the production of several growth factors, such as VEGF, transforming growth factor (TGF)-β1, fibroblast growth factor (FGF), which play a pro-angiogenic and/or pro-fibrogenic role in this process. Secondly, the progression of liver fibrosis increases vascular resistance in portal tracts and sinusoids and results in tissue hypoxia. Tissue hypoxia is a key switch to increase the expression of hypoxia inducible factor (HIF)-1α and VEGF [Fernández et al., 2009; Pinzani et al., 2011]. This pathological angiogenesis creates abnormal vasculature in the liver, such as septal shunts: portal vein-to-terminal hepatic vein shunt; hepatic artery-to-terminal hepatic vein shunt, which leave hepatic parenchyma deprived of significant blood flow. Besides angiogenesis, repetitive liver damage also leads quiescent stellate cells into activated myofibroblasts. This activation of myofibroblasts induce increased collagen synthesis and decreased matrix metalloproteinases (MMPs) production followed by organ fibrosis.

Fibrogenesis itself is thought to contribute to hepatocarcinogenesis, among others by paracrine interactions between stromal cells and tumorigenic cells [Zhang and Friedman, 2012]. Activated stellate cells not only produce growth factors such as hepatocyte growth factor (HGF), interleukin (IL)-6 and Wnt ligands that facilitate a growth friendly environment for proliferating hepatocytes but also promote

angiogenesis through secretion of Angiopoietin-1 [Friedman, 2008; Taura et al., 2008].

HCC develops in the above described environment of active angiogenesis, fibrogenesis and an altered hemodynamic milieu which favor hypoxia [Hernandez-Gea et al., 2013]. During hepatocarcinogenesis increased need for oxygen and nutrients will increase angiogenic activity. According to recent insights tumor angiogenesis is not only regulated by cancer cells but also sustained by stromal cells in the tumor microenvironment [Hanahan and Coussens, 2012]. It is from this perspective that we performed the study in chapter 3 in which we investigated whether angiogenic activity in the tumor and in the adjacent tissue is associated with micro-VI. In an environment with active angiogenesis, neovessel formation may yield abnormal vessels, in particular leaky permeable vessels that are incompletely covered by pericytes which facilitate tumor intravasation [Hanahan and Coussens, 2012].

In **chapter 3**, we investigated the angiogenic profile of primary HCC and peritumoral cirrhotic but non-cancerous liver in relation to the presence or absence of micro-VI. We found increased expression of placental growth factor (PIGF) in the peritumoral tissue of micro-VI positive HCC as compared with the peritumoral tissue of the micro-VI negative HCC. In addition, in the peritumoral tissue of the micro-VI positive HCC, PIGF and vascular endothelial growth factor receptor (VEGFR)-1 expression was upregulated compared with its corresponding HCC. In the micro-VI negative group this discrepancy was not observed. These findings indicate the significance of the changes in the micoenvironment of the tumor which is the location of micro-VI in HCC.

PIGF stimulates endothelial cell growth, survival and migration in pathological condition but PIGF is not involved in normal vascular development and maintenance. PIGF can potentiate the angiogenic effect of VEGF mediated signaling by binding to VEGFR-1 and displacement of VEGF from VEGFR-1 to VEGFR-2 [Carmeliet et al., 2001]. Overexpression of PIGF results in increased tumor vascularization consisting of abnormal blood vessels with incomplete coverage by pericytes and an irregular basement membrane [Adini et al., 2002] whereas blockage of PIGF inhibits tumor vessel arterialization and abnormalization as observed in experimental HCC. The latter process also impedes tumor growth without significant effects on vessel density [Van de Veire S et al., 2010]. Therefore it is conceivable that the peritumoral profile of increased PIGF and VEGFR-1 gene expression may stimulate generation of abnormal vessels that are permissive to tumor intravasation due to its abnormal architecture. Recently, Heindryckx et al demonstrated in an animal model of HCC that anti-PIGF antibody can inhibit neovascularisation and normalize the remaining blood vessels. The authors suggested that this treatment can reduce the prometastatic potential of HCC

[Heindryckx et al, 2013]. Development of new drugs can follow this strategy of normalizing the abnormal structures within the tumor microenvironment [Jain, 2013].

Epithelial-mesenchymal transition and microvascular invasion

EMT was originally defined by the formation of mesenchymal cells from epithelial cells in a physiological embryonic process, and has now been linked to fibrosis and cancer progression. According to the involvement of EMT in these three different biological settings, EMT was classified into three subtypes by Kalluri and Weinberg [Kalluri and Weinberg, 2009].

Type 1 EMT is observed in various phases of embryonal development and organ maturation, and causes neither fibrosis nor uncontrolled cancer invasion. For instance, the primitive epithelium gives rise to primary mesenchyme through the formation of primitive streak generated in the epiblast layer [Hay, 2005; Lim and Thiery, 2012].

Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis, which are presumably the consequence of inflammation. It has been shown that some activated fibroblasts/myofibroblasts are derived from pre-exsiting epithelium via EMT, and are able to produce collagen-rich extracellular matrix in the development of organ fibrosis [Carew et al., 2012]. A big difference between Type 1 and Type 2 EMT is that type 2 EMT is pathogenic and can cause organ dysfunction as long as the causes of inflammation or tissue damage are not removed.

Type 3 EMT occurs in the process of epithelial cancer progression in which carcinoma cells lose their epithelial characteristics and acquire mesenchymal features in order to obtain the ability to invade and to disseminate [Meng and Wu, 2012]. Due to the increased motility, these cells are able to detach from the basement membrane, invade the tumor stroma and blood vessel to enter the circulation. To metastasize, the cancer cells have to exit the blood vessels to form micro- and macro metastases at suitable sites. The latter steps may require a reverse process, mesenchymal-epithelial transition (MET), to enable the cancer cells to colonize and grow at the new sites [Meng and Wu, 2012]. This complex and multistage process does not only induce invasiveness, but the acquisition of mesenchymal cell properties goes along with survival enhancing capabilities by evasion of apoptosis, cellular senescence, dependence on oncogenes and immune defense [Tiwari et al., 2012]. Of note, these competences are in line with the recent insight that carcinoma cells that undergo EMT contain cancer stem cell (CSC) properties [Tiwari et al., 2012; Meng and Wu, 2012]. EMT and cancer progression is orchestrated by cascades of signaling pathways involving a complex cross talk between these pathways. In short, following proper stimulatory signals several factors such as TGFβ, Wnt, insulin-like growth factor (IGF), FGF, HGF,

platelet-derived growth factor (PDGF) and epithelial growth factor (EGF) may activate signaling pathways that result in expression of several EMT inducing transcriptional factors including Snail -1, Snail-2, Zeb-1, Zeb-2 and Twist [Tiwari et al., 2012]. Another group of important players in this EMT orchestra is the micro-RNAs. Several micro-RNAs are involved in EMT by positively or negatively influencing the expression of ligands, receptors, signaling pathways and transcription factors [Tiwari et al., 2012; Meng and Wu, 2012].

Transformation of epithelial cell phenotype into a mesenchymal one is characterized by loss of epithelial cell markers, e.g. E-cadherin, cytokeratins and acquisition of mesenchymal ones such as N-cadherin, α5β6 integrin and nuclear β-catenin [Tiwari et al., 2012]. The loss of E-cadherin expression by carcinoma cells is one of the effects of EMT and an initial step of invasion and it has been reported in several types of invasive carcinomas, including HCC [Thiery et al., 2009]. E-cadherin is a vital cell-to-cell adhesion molecule that strengthens the epithelial cells in cell sheets and maintains the cellular quiescence in these sheets [Schmalhofer et al., 2009]. Loss of E-cadherin may result from gene mutation leading to production of a defective protein, an aberrant posttranslational modification or increased proteolysis, regulated by several of the abovementioned pathways [Tiwari et al., 2012]. In HCC, increased expression of the transcription factors Twist and Snail, in association with repression of E-cadherin and the presence of a CSC population has been reported [Yang et al., 2004; Cano et al., 2000]. In HCC, cytokeratin (CK) 19 and epithelial cell adhesion molecule (EpCAM) are regarded as CSC markers since CSCs share their markers with somatic stem cells of the original tissue in the different types of cancers [Yamashita et al., 2009; Kim et al., 2011].

In **chapter 3,** we found decreased E-cadherin expression within the micro-VI positive HCC at both gene and protein levels compared with the micro-VI negative ones. However, we did not find a correlation between the existence of micro-VI and the expression of the hepatic stem-cell markers CK19 or EpCAM in HCC. In the non-cancerous adjacent liver, both CK19 and EpCAM are expressed by bile ductules, which possibly harbor bipotent liver progenitor/stem cells, [Schmelzer et al, 2006; Zhang et al., 2008; Gouw et al., 2011].

The relationship between EMT and CSC are not fully understood. One possible link is the fact that the cells that undergo EMT contain CSC properties, and such cells that possess tumorigenicity may play a critical role in the proliferation of cancer cells at the distant site as well as in the progression of cancer at the primary site during the early phase [Scheel et al., 2012]. Other factors that have been identified recently as positive inductors of EMT and micro-VI in HCC are stress-activated protein kinase (SAPK) interacting protein 1 (SIN1) and sirtuin 2 (SIRT2), each operating through different pathways. Xu et al showed a significant correlation

between increased SIN1 expression and poor prognosis and clinicopathological parameters related to prognosis, such as tumor numbers and venous invasion. Moreover, it has been shown that SIN1 facilitates EMT through Akt activation followed by the upregulation of Snail [Xu et al., 2013]. Chen et al also reported that the upregulation of SIRT2 was significantly correlated with poor overall survival and micro-VI, and demonstrated that SIRT2 expression levels are associated with mortality and invasiveness of HCC cells and with EMT through protein kinase B/glycogen synthase kinase-3β/β-catenin signaling [Chen et al., 2013]. Autophagy, which is an intracellular process for the degradation of unnecessary cellular components, has also been reported to positively influence invasion in HCC through stimulation of EMT via induction of the TGFβ/Smad pathway [Li et al., 2013].

Tumor characteristics that influence microvascular invasion

Tumor characteristics usually include the gross appearance, size, the number of tumor nodules and the cellular morphology. All these different phenotypic features have been found to be associated with micro-VI as reviewed in **chapter 2.** The gross appearance of HCC tumor nodules is predominantly studied and applied as a predictive factor for micro-VI in Asian countries. The Liver Cancer Study Group of Japan classified nodular HCC into 3 subtypes depending on the contours of the nodules and based on the macroscopic appearance of the resected specimens. The simple nodular type is a distinct nodule with a smooth outline which frequently consists of a fibrous capsule. When such a nodule shows extranodular growth it represents the second type. The third type consists of coalescing nodules, the confluent multinodular type [Liver Cancer Study Group of Japan, 2009]. The latter type has the strongest association with micro-VI [Hui et al, 2000; Sumie et al., 2008].

Several studies reviewed in **chapter 2** showed variable results on the association between micro-VI with these tumor characteristics but in general an increase in the prevalence of micro-VI parallels increases in tumor size, number of tumor nodules and higher tumor grade. In our study we found an association between micro-VI and higher tumor grade (**chapter 3)** but not with the other tumor characteristics. The variable results may be explained by factors such as sample size of the study, the robustness of the method to detect micro-VI and the grading of the tumor. However, all mentioned tumor characteristics are in fact surrogate markers of the growth potential of the tumor. Size, contours and number of nodules reflect the mode and pace of tumor growth whereas tumor grading identifies an aggressive or less aggressive cell population. Higher grade tumors consist of less differentiated tumor cells associated with more aggressive behavior, hence increasing the chances of invasion. Apart from assessment of histomorphological features based on the HE staining that is routinely applied for tumor grading,

identification of other cellular features, such as CSC characteristics can be done by specific immunophenotyping. The presence of CSC populations in HCC has been reported in a number of studies, in which they were identified by various types of cell surface markers, e.g., CK19, CD133, CD90, EpCAM, and CD13. [Yin et al., 2007; Yang et al., 2008; Yamashita et al., 2009; Haraguchi et al., 2010]. CK19 is regarded as a marker for bipotent hepatic progenitor/stem cells which have the capability to differentiate to either hepatocytes or cholangiocytes. Among these markers, immunophenotyping using EpCAM and CK19 have been well studied, probably because antibodies to detect EpCAM and CK19 are robust and easily obtained. The presence of both EpCAM positive and CK19 positive tumor cells in HCC is associated with vascular invasion and poor prognosis and is thought to be associated with their CSC features. CSC are characterized by the following abilities: self renewal with or without differentiation; high tumorigenicity; and chemoresistance, all of which are functions that increase the capacity for tumor recurrence and/or metastasis.

Among patients with small HCC, the prognostic role of micro-VI is still controversial. Recently Shindoh et al have reported that the presence of micro-VI does not affect patients' prognosis in HCC up to 2cm [Shindoh et al., 2013]. On the other hand, Nakashima et al reported a higher frequency of micro-VI in confluent multinodular type and single nodular type with extranodular growth than in single nodular type among HCCs less than 3cm [Nakashima et al., 2003]. It is possible that there are tumor subgroups that are likely to have micro-VI and early recurrence due to a different biological behavior e.g. the confluent multinodular type HCC. This type is characterized by a lack of capsule which may faciltate invasion. This speculation requires further studies regarding the relationship between gross type and growth behaviour, especially in small HCCs.

The role of ductular reaction in microvascular invasion

The results of the studies in **chapter 3** indicate the importance of the peritumoral compartment. In **chapter 4** thus we performed further investigations in this area. Using laser microdissection we isolated the peritumoral fibrous septa (PT septa) and perinodular cirrhotic septa collected from areas distant from the tumor (D septa). Gene expression studies of genes related to angiogenesis, EMT and liver progenitor cells such as EpCAM and CK19 revealed a significant decrease of EpCAM and E-cadherin in PT septa of micro-VI positive HCC septa compared to its D-septa counterpart. This difference was not found in the micro-VI negative HCC. Since both EpCAM and E-cadherin can only be expressed by ductular cells as these are the only epithelial structures in the fibrous septa, we assumed that these different gene expression levels of EpCAM and E-cadherin may result from the decreased number of ductules in PT septa which would be in accordance with the

results of Lennerz et al [Lennerz et al., 2011]. Semiquantitative scoring of the ductular structures in PT septa and D septa using EpCAM immunohistochemistry (chapter 4) confirmed the gene expression results that there is a decrease of EpCAM positive ductules in micro-VI positive PT septa compared with the corresponding D septa, although the difference did not reach statistical significance (P=0.09) which was probably due to the small sample size of 11 samples. To validate the results of this first cohort, a similar quantitative study was performed on a second cohort of 25 samples labeled with EpCAM and scored without prior knowledge of the status of micro-VI. A significant lower numbers of EpCAM positive ductules was found in the PT septa of micro-VI positive HCC in comparison with the corresponding D septa (P=0.002). This pattern was not found in the micro-VI negative HCC. This finding is of potential value to be applied as a predictive factor for micro-VI in HCC. A similar quantification can be applied in biopsy specimens containing sufficient peritumoral septal areas and fibrous septa distant from the tumor. Robust validation studies are however necessary prior to implementation of this analysis in daily clinical practice.

Ductular reaction is a reactive process which arises in diseased livers, at the interface of portal and parenchymal compartments during liver damage and regeneration [Gouw et al., Hepatology 2011]. Recently ductular reaction has been found to play a role in the transition of dysplastic nodules in cirrhotic livers into HCC. Lennerz et al documented diminishing numbers of CK19 positive perinodular ductular structures in parallel with progression of intranodular hepatocytes into HCC. The disappearance of the ductular cells was neither due to necrosis nor apoptosis. Concurrent with these changes there was an increase of cells expressing EMT markers [Lennerz et al., 2011]. These findings are compatible with EMT as part of cancer progression as has been addressed in the previous paragraphs. The fact that perinodular structures undergo such profound changes during hepatocarcinogenesis corroborates our findings in **chapters 3 and 4** that the peritumoral tissue, as part of the tumor microenvironment plays an important role in HCC progression which includes micro-VI.

Part II. Systemic therapy of HCC

Despite the recent progress in the management of HCC, the majority of HCC is diagnosed at the intermediate or advanced stage [EASL, 2012] representing the stages of BCLC-B, C, or more. Patients within these categories are not eligible for potentially curative radical therapies. Therefore, the development of effective systemic therapy for HCC is needed. As mentioned in the general introduction part, sorafenib is the first, and so far the only systemic agent with proven survival benefit among BCLC-C patients in several large randomized controlled trials (RCTs) [Llovet et al., 2008; Cheng et al. 2009]. This clinical success of sorafenib in HCC evoked a trend of molecular targeted drugs for HCC. Dozens of molecular targeted drugs for HCC are currently being assessed in clinical trials, which are broadly classified as either monoclonal antibody (MAb) or small molecule tyrosine kinase inhibitors (TKI) [Villanueva and Llovet, 2011]. MAbs target specific antigens found on the cell surface which are closely related to tumor progression. TKIs interfere with the enzymatic activity of the targeted tyrosine kinase. For instance, Sorafenib inhibits several receptor tyrosine kinases, such as VEGFR and platelet-derived growth factor receptor (PDGFR) located on the cell membrane, and intracellular kinase pathways.

The role of interferon therapy

By the end of 2012 after the initial success of sorafenib, 4 new molecular targeted drugs: sunitinib; brivanib; linifanib and erlotinib, were assessed by phase 3 clinical trials, and 4 more drugs: regorafenib; everolimus; ramucirumab; tivantinib, are now in phase 3 clinical trials [Villanueva, 2013]. Notably, the main targets of 5 out of these 8 new drugs include VEGFR, PDGFR and Tie-2 which is the specific receptor for Angiopoietins. Thus angiogenesis is regarded as an important target for HCC therapy. However, 4 drugs that have already been evaluated did not show positive results and 3 of the 4 were those targeting these angiogenesis-related tyrosine kinase receptors. This fact requires a reconsideration of the strategy of molecular-targeted cancer therapy. One possible approach to be considered is a molecular approach of patient selection. For instance, transtuzumab which inhibits HER2/neu receptor is administered to patients with HER2-positive breast cancer [Slamon et al., 2001], and cetuximab, which interferes with EGFR, to those with EGFR- expressing colorectal cancer [Cunningham et al., 2004]. In addition, during clinical studies with cetuximab, it was shown that the absence of the K-ras mutation is a favorable factor for the response [Karapetis et al., 2008]. Similary, it is known that gefitinib and erlotinib which also inhibit EGFR are effective in patients with EGFR-mutated lung adenocarcinoma [Paez et al., 2004]. Another approach is to target other molecular players, e.g. mutated genes that are critically involved in

hepatocarcinogenesis. For instance, an abnormal translocation between chromosome 9 and 22 and the consequent continuously activated Bcr-Abl tyrosine kinase underlie the development of chronic myelogenous leukemia (CML). Therefore, imatinib which inhibits Bcr-Abl tyrosine kinase can show dramatic effects on patients with CML [Goldman and Melo, 2003]. Although such a chromosomal translocation is rare among malignant epithelial neoplasms, EML4-ALK fusion was detected in some lung adenocarcinoma [Soda et al., 2007]. Following this novel finding, anaplastic lymphoma kinase (ALK) inhibitor crizotinib was readily approved as a first line drug for patients with ALK positive lung cancer [Shaw et al., 2011]. Patients selection seems important in the success of molecular-targeted drug. Such an approach for HCC requires discovery and validation of such drugs.

Apart from molecular targeted drugs, conventional anti-cancer drugs for HCC also should be evaluated as potential drugs that might give some add-on effects to sorafenib. Although there are few drugs that have shown such an effect against HCC, IFNs might be candidates. IFNs are cytokines that are produced by host cells, such as leukocytes, in response to inflammation. Since IFNs possess antiproliferative activity, IFN therapy is used to treat patients with malignant melanoma, acquired immunodeficiency syndrome-related Kaposi's sarcoma, and some hematopoietic malignancie [Garbe et al., 2011; Pestka et al., 1987; Jonasch and Haluska, 2001]. In the field of liver disease, IFNs are commonly used to treat patients with chronic viral hepatitis because of their antiviral activity and immunoregulatory effect. Especially, for the patients with hepatitis C infection, combination therapy of pegylated IFN (PEG-IFN) and ribavirin, with or without telaprevir, is standard [Ghany et al., 2009; Jacobson et al., 2011]. Two decades ago it was shown by RCT conducted by Lai et al for the first time that IFN-α therapy can prolong patient survival in inoperable HCC [Lai et al., 1993]. In addition, systemic IFN therapy can improve survival in advanced HCC in combination with hepatic intraarterial cisplatin infusion compared with hepatic intraarterial infusion (HAI) monotherapy [Chung et al., 2000]. Some Japanese researchers also showed effects of combination therapy of systemic IFN administration and hepatic intraarterial 5-fluorouracil (5-FU) infusion [Obi et al., 2006], and RCTs regarding IFN therapy combined with HAI are ongoing.

Thus IFNs have been used for patients with diseased liver, and have potential benefits in patients with HCC. It is worthwhile to study the add-on effect of IFNs in HCC treated with sorafenib.

In **chapter 5 and 6**, we investigated the antitumor effects of PEG-IFNs with or without sorafenib on HCC cells *in vitro* and *in vivo*. Firstly, we found that PEG-IFN monotherapy can decrease tumor progression *in vivo* and is more effective than non-pegylated forms of IFN. Secondly, although the effects of therapy differed in cell lines, combination therapy of sorafenib and PEG-IFN can induce better effects

than monotherapy. It is also believed that IFNs have chemopreventive effects against the development of HCC to some degree. However, two large RCTs, which were conducted by the HALT-C and EPIC groups, failed to prove that long term maintenance PEG-IFN therapy can reduce the incidence of HCC [Di Bisceglie et al., 2008; Lok et al., 2009; Bruix et al., 2011]. On the other hand, there are several reports that showed survival benefit or the reduction of recurrent HCC of IFN therapy after initial therapy [Sakaguchi et al., 2005; Nishiguchi et al., 2005]. There are also some reports that showed IFN therapy reduced the occurrence of HCC among particular patient groups, e.c. patients with HBV infection or those who underwent curative resection of HBV-related HCC [Miyake et al., 2009; Qu et al., 2010]. Again, patient selection is crucial for the success of IFN therapy. In 2009, three different groups reported that genetic variation in IL28B encoding IFN-λ-3 is associated with response to PEG-IFN and ribavirin therapy for chronic HCV infection [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. The association of this genetic variant with response to IFN therapy for HCC should be studied.

In this thesis, we performed studies on two issues that influence the prognosis of HCC, micro-VI (part I) and sensitivity of HCC cells to IFN (part II). In **chapter 2** we reviewed the current status of methods to predict micro-VI preoperatively and concluded that currently there is no reliable method to predict micro-VI before surgery. The studies in **chapters 3 and 4** were both performed to establish a profile of micro-VI positive HCC that may yield a predictive factor of micro-VI. Results of these studies indicated the importance of peritumoral tissue as a compartment that contains changes related with micro-VI. This was represented partly in altered angiogenic characteristics as witnessed by upregulation of PIGF and VEGFR-1 expression and partly in decreased numbers of ductular structures that probably undergo phenotype switching during progression of HCC as part of cancer related EMT. The decreasing numbers of ductules may serve as a predictive factor of micro-VI in tumor biopsies.

The studies in part II evaluated PEG-IFNs as a promising drug combined with sorafenib. As 70% to 80% of patients with HCC are diagnosed at the stage where there is no curative treatment option, effective systemic therapy options are still sought for. In **chapter 5 and 6**, we demonstrated the anti-proliferative effect of PEG-IFNs with or without sorafenib in various HCC cell lines. Sensitivity to each combination varied leading us to conclude that molecular tumor profiling will be necessary to improve the sensitivity of therapies based on the matching of the tumor with certain drugs or combination of drugs.

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Chapter 2

Markers for microvascular invasion in hepatocellular carcinoma: Where do we stand?

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It is well known that hepatocellular carcinoma (HCC) commonly involves the local branches of portal and/or hepatic veins and causes a tumor thrombus even at a relatively early stage. Vascular invasion is classified as macrovascular invasion, which is grossly recognizable (mostly in large to medium vessels), or microvascular invasion (MVI), which can be identified only by microscopic observation (mainly in small vessels such as portal vein branches in portal tracts, central veins in noncancerous liver tissue, and venous vessels in the tumor capsule and/or noncapsular fibrous septa).

Although macroscopic vascular invasion in major vessels (and satellite nodules) is known to be a marker of poor outcomes after liver transplantation (LT) for HCC and is regarded as a contraindication for LT, the significance of MVI as a predictor of poor outcomes is still controversial. The controversy concerns the extent to which MVI (if it is identifiable before surgery) is a contraindication for LT, even though we know that only a minority of patients with MVI will experience HCC recurrence.

MATERIALS AND METHODS

We searched the MEDLINE database (2002-2010) to determine the significance of MVI to the outcomes of LT for HCC; we used the keywords hepatocellular carcinoma, HCC, microvascular invasion, liver transplantation, and liver resection. We also performed a full manual search of the bibliographies of selected publications and included 4 additional publications from earlier years. Publications were included if they contained data on MVI and its relationship with tumor characteristics and/or prognostic data. The search resulted in a total of 48 relevant publications.

The publications were then ranked according to the classification proposed by the Oxford Centre for Evidenced-Based Medicine.

RESULTS

Question 1. Is There Any Correlation Between MVI and Tumor Characteristics?

Many published studies have found that the presence of MVI is closely related to the tumor size, number, and histological grade [1-8] (Table1). Esnaola et al. [1] studied MVI in 245 HCC patients who underwent surgical resection and fulfilled the Milan criteria, and they found that 33% of their patients had histopathological evidence of MVI. Patients with tumors larger than 4 cm were 3 times more likely to

have MVI than those with tumors measuring 4 cm or less. Patients with poorly differentiated or undifferentiated tumors were 6 times more likely to have MVI than those with well-differentiated tumors. In comparison with patients with well-differentiated tumors, patients with multiple tumors were 2 times more likely to have MVI, and patients with moderately differentiated tumors were 2.6 times more likely to have MVI. Others have also reported that the presence of MVI is closely related to a larger tumor size and a lower histological grade (Table 1).

Table 1. MVI and Tumor Characteristics

	Size (cm)	Number	Histological grade
Esnaola et al. [1]	>4	NS	Moderately to poorly differentiated
Shah et al. [2]	>3.8	>3	NS
Parfitt et al. [3]	>3	>3	Moderately to poorly differentiated
Löhe et al. [4]	>5	NS	Moderately to poorly differentiated
Jonas et al. [5]	3~5	2~3	Moderately to poorly differentiated
Bhattachrijya et al. [6]	>4	3	Moderately to poorly differentiated
Shirabe et al. [7]	>2-4	multiple	Moderately to poorly differentiated
Pawlik et al. [8]	>5	multiple	High grade (moderately to poorly differentiated)

Relationship Between MVI and the Gross Type

The Liver Cancer Study Group of Japan proposed a gross classification scheme for resected HCC in 1997 [9], and it has been widely used in Japan. In the Liver Cancer Study Group of Japan classification scheme, nodular tumors are divided into the following subclasses: (1) a simple nodular type (a distinctly nodular tumor that frequently has a capsule), (2) a simple nodular type with extranodular growth (a single nodular tumor with varying degrees of tumor growth beyond the tumor capsule), and (3) a confluent multinodular type (a confluence of several minute to small nodules; see Fig. 1).

Since the Liver Cancer Study Group of Japan classification was proposed, it has been reported that the rate of MVI is closely related to the gross type.10-12 In a study of 65 resected HCCs, Hui et al.[10] reported that the MVI rates were 17% for the simple nodular type, 25% for the simple nodular type with extranodular growth, and 53% for the confluent multinodular type ($P < 0.03$ for the simple nodular type versus the simple nodular type with extranodular growth, $P < 0.02$ for the simple nodular type versus the confluent multinodular type). In a study of 110 resected HCCs, Sumie et al.[11] also reported a significantly higher prevalence of MVI for the simple nodular type with extranodular growth (72%) and the confluent multinodular type (84%) in comparison with the simple nodular type (20%, $P < 0.001$). They stressed that the gross classification of the simple nodular type with extranodular growth or the confluent multinodular type was an independent predictor of MVI in their study [hazard ratio (HR) = 11.81, 95% confidence interval (CI) = 3.93-37.80, P

< 0.001].

In a large series, Kaibori et al.[13] confirmed that a large tumor size was a preoperative predictor of MVI. Unfortunately, the MVI group included patients with macrovascular invasion.

Similar results were obtained by Shirabe et al.[14] for patients undergoing living donor LT. An independent predictor of poor recurrence-free survival was preoperative type 3 HCC (a contiguous multinodular type with a large tumor size, a poor histological grade, and a high incidence of MVI and multiple tumors).

Notably, tumor cell invasion of the portal vein is observed in 27% of cases with early HCC (ie, up to 2 cm) of a distinctly nodular type, and minute intrahepatic metastases in the vicinity of the tumor are present in 10%. These features are not observed in small HCCs of an indistinct nodular type [15].

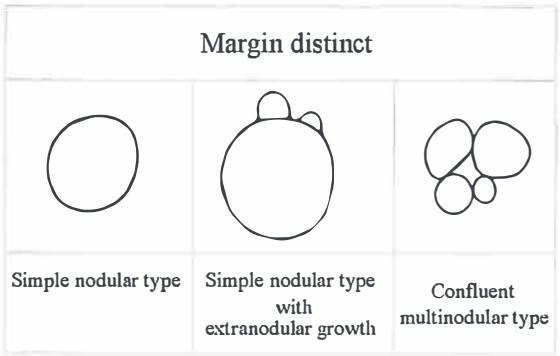

Figure 1. Gross classification scheme for HCC by the Liver Cancer Study Group of Japan. Reprinted with permission from *General Rules for the Clinical and Pathological Study of Primary Liver Cancer* [9]. Copyright 2010, Kanehara & Co., Ltd. Tokyo, Japan.

Comment

The majority of studies have shown a close correlation between MVI and tumor characteristics. Increases in the prevalence of MVI parallel increases in the tumor size, number, histological grade, and gross features. The last are based on the Japanese gross classification system, which is not widely applied in Western countries [9].

In summary, because MVI is a microscopic feature that can be assessed only on the tissue level, MVI cannot be definitely confirmed during the pre-LT stage. However, to a certain extent, the likelihood of the presence or absence of MVI can be predicted during the pre-LT stage on the basis of the tumor size and number, which are assessable with imaging modalities. The histological grade is an additional predictive parameter on the tissue level that can be assessed by pre-LT biopsy, whereas the gross features can be examined only after LT. After LT, MVI is

valuable because of its association with the tumor recurrence rate, which we address later.

Notably, 2 studies have reported that the tumor growth rate or the tumor doubling time is predictive of tumor recurrence after LT [16, 17]. The association between these features and MVI has not been studied, but they merit further attention as additional important tumor characteristics that might be related to MVI.

Question 2. Are There Any Imaging Modalities for Detecting MVI ?

Although macrovascular invasion can be preoperatively detected by conventional imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound, the preoperative imaging determination of MVI is impossible because MVI is a microscopic parameter.

However, Kornberg et al.[18] reported that increased [^{18}F]fludeoxyglucose ([^{18}F]FDG) uptake during positron emission tomography (PET) is predictive of MVI and tumor recurrence after LT. In their study, 42 patients who underwent LT for HCC were examined with PET preoperatively; 16 had positive PET scans, whereas 26 showed no increase in their fludeoxyglucose (FDG) uptake. Five of 6 patients with a poor tumor grade had a PET-positive status, and 14 of 17 patients with MVI (82.4%) had a positive PET finding. Eight of the 16 patients with a PET-positive status (50%) developed HCC recurrence, whereas only 1 of the 26 PET-negative patients did ($P < 0.001$). Furthermore, the PET-negative patients had a significantly better 3-year recurrence-free survival rate than the PET-positive patients. It has been shown that well-differentiated HCC cells exhibit an [^{18}F]FDG metabolism similar to that of normal liver cells, whereas poorly differentiated tumor cells do not. The authors hypothesized that PET scan patterns may have a clinically relevant correlation with the presence of MVI because of the close relationship between MVI and a poor tumor grade. However, there is no evidence that FDG uptake is an independent predictor.

More recently, positive FDG PET results at the time of listing for LT have been found to reflect tumor behavior and to be strongly associated with dropout due to tumor progression. PET could be proposed as an additional tool for the pre-LT assessment of candidates with HCC [19]. Hui et al.[10] tested whether the Japanese gross classification system9 could be used with preoperative dynamic CT (10-mm interval slices). They found that classifications based on CT findings and classifications based on macroscopic observations were concurrent for 46% of their patients. The simple nodular type, the simple nodular type with extranodular growth, and the confluent multinodular type were identified at rates of 62%, 26%, and 40%, respectively.

Comment

So far, conventional imaging modalities have been ineffective for the preoperative detection of MVI. Only 1 study has shown the potential usefulness of PET scans in the prediction of MVI [18]. The preliminary results of an MRI study have shown that the MRI features of HCC do not predict MVI in the transplanted liver [20]. Although the gross classification scheme of the Liver Cancer Study Group of Japan9 is not popular in Western countries, because of the 40% identity rate for dynamic CT findings and the confluent multinodular type (for which there is an MVI prevalence rate of 53%-84%), gross subclassification by dynamic CT merits further study.11 This can also be recommended because of the potential value of PET scans and MVI and the scarcity of data.

Question 3. Are There Any Biochemical Markers for Predicting MVI?

Des-gamma-carboxyprothrombin (DCP), also known as protein induced by vitamin K absence or antagonist II, is an abnormal form of prothrombin, and it has been used as a good diagnostic biomarker for HCC, particularly in Japan [21-23]. It has been reported that high serum levels of DCP reflect the invasiveness of HCC and are related to the histological vascular invasion of cancer cells. Koike et al. [22] prospectively studied 227 patients with HCC who did not show portal venous invasion and who received percutaneous ethanol injection therapy and/or microwave coagulation therapy at the time of their first admission. The patients were followed for a mean of 19 months with ultrasonography every 3 months, with CT scans every 6 months, and with measurements of biochemical parameters such as DCP and alpha-fetoprotein (AFP) every month. A multivariate Cox regression analysis of various factors showing significant correlations with the later development of portal venous invasion revealed that the DCP level was the strongest predisposing factor ($P < 0.001$). Shirabe et al.[7] studied 218 patients who had tumors without any extrahepatic metastases or vascular invasion (according to their preoperative evaluations) and underwent HCC resection. They found that the preoperative DCP level for the patients with MVI was significantly higher than the level for the patients without MVI ($P < 0.049$), and it was an independent predictor of MVI. In another recent study [14], the same authors showed that a preoperative serum DCP level exceeding 300 mAU/m was an independent predictor of poor recurrence-free survival in patients undergoing living donor LT.

In a study of 144 HCC patients treated with living donor LT, Fujiki et al.[23] reported that the incidence of MVI was significantly higher for patients with a DCP level > 400 mAU/mL versus patients with a DCP level ≤ 400 AU/mL.

Eguchi et al.[21] studied 179 patients without macroscopic venous invasion who underwent curative resection for HCC. High preoperative levels of DCP and the gross appearance of the tumor appeared to be strong predictive factors for MVI,

even for tumors less than 5 cm in size. The gross features that were associated with MVI were extranodular growth, multinodularity, and infiltrative growth.

The value of DCP in predicting MVI recurrence should not be compared to the value of DCP in predicting HCC occurrence. Sterling et al.[24] concluded that mild to moderate elevations of the total AFP and DCP levels (but not the AFP-L3 levels) occur frequently in patients with chronic hepatitis C and advanced cirrhosis, but they are related to factors other than HCC and are poor indicators of HCC. They recommended that these biomarkers not be part of routine surveillance protocols.

An increase in the AFP level greater than 15 μg/L/month during the wait for LT has been shown to be the most relevant preoperative prognostic factor for low overall and disease-free survival. An elevated AFP level was shown to be a predictor of a lower recurrence-free survival rate in a large series of 283 transplant patients (odds ratio = 2.88, 95% CI = 1.43-5.80, P = 0.003).25 An increase in the AFP level could be a preoperative pathological marker of tumor aggressiveness [26]. In the past 2 decades, attempts have been made to detect circulating tumor cells in the peripheral blood of HCC patients through the examination of tumor-related markers [27-29]. However, the results are still only probable because of the lack of truly reliable HCC-related markers.

It is likely that new blood markers will be proposed in the future. Recently, the angiopoietin 2 level has been shown to be a predictor of tumor invasiveness in patients with HCC [30].

Comment

The significance of a high serum DCP level as a good biomarker for predicting MVI has been suggested mostly in Japanese studies. Hence, an international, multicenter study is required to assess the precise predictive value of DCP in a broader group of HCC populations.

Furthermore, other predictive parameters should be evaluated. Reports on factors in HCC tissue that are associated with MVI at protein and gene expression levels are emerging. Liver-intestine cadherin has been reported to be correlated with MVI in hepatitis B virus–related HCC [31]. A genetic signature of microvascular invasiveness has also been reported in a genome-wide analysis [32]. The potential of these factors as future biomarkers merits further studies.

The use of an artificial neural network to predict the tumor grade and MVI on the basis of noninvasive variables is notable [33]. An artificial neural network and logistic regression models were based on a training group of 175 randomly chosen patients and were tested on 75 patients. In the testing group, the artificial neural network correctly identified 93.3% of the tumor grades (k = 0.81) and 91% of the MVI cases (k = 0.73). The logistic models correctly identified 81% of the tumor grades (k = 0.55) and 85% of the MVI cases (k = 0.57). In this study, many clinical,

radiological, and pathological factors were taken into account, but the DCP level and the body mass index were not. Surprisingly, in a study of 138 consecutive patients who underwent surgery at Columbia University Medical Center from January 1, 2002 to January 9, 2008, Siegel et al.[34] found that patients with a body mass index > 30 kg/m2 had MVI at rate of 40%, whereas patients with a body mass index < 25 kg/m2 had MVI at a rate of 14%.

Question 4. Is MVI Related to the LT Outcome?

The relationship between MVI and LT outcomes is still a matter of debate. The majority of studies favor the view of a relationship between MVI and poor posttransplant outcomes [1-11, 35-53]. Many studies have reported a close correlation between the presence of MVI and a poor prognosis after LT; Bhattacharjya et al.[6] performed a prospective study of 30 patients treated with LT for relatively small HCCs (range = 6-75 mm). They found that the tumor size and the presence of multifocal tumors did not influence survival, but MVI, which was more common with larger tumors (38% with tumors < 4 cm and 60% with tumors > 4 cm, P < 0.01), influenced survival. Cescon et al.[25] reported a single-center series of 283 patients who underwent transplantation for HCC between 1997 and 2009; a Cox proportional hazards model showed that MVI was a predictor of lower recurrence-free survival (odds ratio = 4.82, 95% CI = 1.87-12.41, P = 0.001). The fact that the Milan criteria did not appear to be prognostic may not be surprising because 89% of the patients fulfilled the up-to-7 criteria at the time of orthotopic LT.

In a series of 51 patients undergoing LT for HCC, Choi et al.[37] retrospectively analyzed 5 cases of incidental HCC with a mean size of 1.16 cm, no MVI, and good to moderate tumor differentiation. They found no recurrence of HCC in a mean follow-up period of 14 months. Lai et al.[47] reported that only MVI and exceeding the University of California San Francisco criteria were independent risk factors for recurrence in a multivariate analysis. However, many have reported that a combination of a larger tumor size, a higher histological grade (less differentiation), and MVI is the strongest factor related to recurrence and a poor prognosis after LT. Jonas et al.[5] found the presence of vascular invasion and the histological grade to be the only statistically significant independent predictors of poor survival after LT. In a study of 69 patients with HCC who underwent LT, Plessier et al.[54] reported that MVI was significantly correlated with the presence of satellite nodules (P < 0.02) and a poor prognosis.

On the other hand, a few studies have not been able to correlate poor results with MVI, although macrovascular invasion has been related to bad outcomes. Lee et al.[49] followed 38 patients after LT for HCC for a mean period of 17.7 months (range = 4-30 months), and they found that the number of tumor nodules and the presence of MVI did not affect tumor recurrence. Fan et al.[55] studied 1078

patients who underwent orthotopic LT and found no correlation between MVI and overall or disease-free survival. Kornberg et al.[46] documented MVI as a risk factor for tumor recurrence after LT. A poor tumor grade (HR = 21.8, 95% CI = 4.9-95.3, P < 0.001) and MVI (HR = 14.1, 95% CI = 1.4-147.1, P = 0.027) were identified as independent risk factors for reduced recurrence-free survival after LT.

Although the risk of recurrence is higher in patients with MVI, not all patients with MVI will experience recurrence. This has to be balanced with macrovascular invasion and satellite nodules (all detected by imaging techniques), which are contraindications for LT because of the high incidence of recurrence.

Table 2 summarizes the publications documenting several types of tumor features, biomarkers, and imaging modalities. Table 3 summarizes the publications specifically documenting MVI and several of these issues, and available correlations are included.

Table 2. Overview of Studies Several Tumor Characteristics

	Patient (n)		Tumor Characteristics						
Reference	analyzed	Meeting Milan criteria	size	n	grade	AFP	DCP	Radiol-ogical image	Gross classifi-cation
Esnaola et al. [1]	245	245	+	+	+	+	-	-	-
Shah et al. [2]	154	154	+	+	+	-	-	-	-
Parfitt et al. [3]	75	50*	+	+	+	+	-	-	-
Löhe et al. [4]	97	47*	+	+	+	-	-	-	-
Jonas et al. [5]	120	120	+	+	+	-	-	-	-
Bhattacharjya et al. [6]	30	30	+	+	+	-	-	-	-
Shirabe et al. [7]	218	NM	+	+	+	+	+	-	-
Pawlik et al. [8]	1073	NM	+	+	+	+	-	-	-
Hui et al. [10]	65	65*	+	-	+	-	-	-	+
Sumie et al. [11]	110	110*	+	+	+	+	+	-	+
Nakashima et al. [12]	209	209*	+	-	+	-	-	-	+
Kaibori et al. [13]	434	NM	+	+	+	+	+	-	-
Shirabe et al. [7]	119	65	+	+	+	+	+	-	+
Cucchetti et al. [16]	62	NM	+	+	+	+	-	-	-
Kornberg et al. [18]	42	20*	+	+	+	+	-	+	-
Eguchi et al. [21]	179	NM	+	+	-	+	+	-	+
Koike et al. [22]	227	NM	+	+	-	+	+	-	-
Fujiki et al. [23]	144	79	+	+	+	+	+	-	-
Cescon et al. [25]	283	208*	+	+	+	+	-	-	-
Vibert et al. [26]	153	99	+	+	+	+	-	-	-
Ding et al. [31]	255	NM	+	+	+	+	-	-	-
Tanaka et al. [32]	59	NM	+	+	+	+	+	-	+
Cucchetti et al. [33]	250	190*	+	+	+	+	-	-	-

Siegel et al. [34]	138	NM	+	-	-	+	-	-	-
Roayaie et al. [35]	131	NM	+	+	+	+	-	-	-
Cucchetti et al. [36]	204	NM	+	+	+	+	-	-	-
Choi et al. [37]	5	NM	+	+	+	+	-	-	-
Dudek et al. [38]	55	48*	+	+	+	-	-	-	-
Plessier et al. [54]	69	69	+	+	-	+	-	-	-
Mazzaferro et al. [39]	1556	444*	+	+	+	-	-	-	-
Yao et al. [40]	70	46*	+	+	+	+	-	-	-
Coelho et al. [41]	45	NM	+	+	+	+	-	-	-
Park et al. [42]	213	176*	+	+	+	+	-	-	-
Vivarelli et al. [43]	139	113*	+	+	+	+	-	-	-
Silva et al. [44]	257	231	+	+	+	+	-	-	-
Suh et al. [45]	19	0	+	+	+	+	-	+	-
Kornberg et al. [46]	60	27*	+	+	+	+	-	-	-
Lai [47]	85	59*	+	+	+	-	-	-	-
D'Amico et al. [48]	479	272*	+	+	+	+	-	-	-
Lee et al. [49]	38	21*	+	+	-	-	-	-	-
Bargellini et al. [53]	33	0	+	+	+	+	-	+	-
Fan et al. [55]	1078	394*	+	+	+	+	-	-	-

*confirmed histopathologically

Table 3. Correlation between microvascular invasion and tumor characteristics

	Tumor characteristics						
Reference	size (cm)	number	grade	AFP	DCP	Radiological Image	Gross classification
Esnaola et al. [1]	**+ >4**	+	**+ poor/undiff**	NS			
Shah et al. [2]	+ >3.6	+ >3.8	NS				
Parfit et al. [3]	+ >5						
Löhe et al. [4]	**+ >5**	NS	+ intermediate/ high grade				
Jonas et al. [5]	**+ >5**	+	**+ Grade 2/3**				
Bhattacharjya et al. [6]	+ >4	NS	+ mode/poor				
Shirabe et al. [7]	+	NS	+	NS	+		
Pawlik et al. [8]	+	**+ multiple**	**+ high grade**	**+ >=1000 ng/ml**			
Hui et al. [10]							+ CM
Sumie et al. [11]	+	NS	+	NS	NS		**+ CM,** IM
Nakashima et al. [12]							+ SNEG, CM
Kaibori et al. [13]	**+ >5****	NS	**+ poor**	NS	**+ >=200mAU/ml**		
Shirabe et al. [14]							+ CM
Kornberg et al. [18]	NS	NS	+ poor	NS		**+ PET positive**	
Eguchi et al. [21]	**+ >5**	+		+	+		**+ SNEG, CM, Inf**
Fujiki et al. [23]	NS	NS		+ >=800 ng/ml	**+ >=400mAU/ml**		
Vibert et al. [26]	**+ >3**	NS		**+ rapid progre-ssion*****			
Cucchetti et al. [33]	+	+		+			
Siegel et al. [34]	NS			+			
Silva et al. [44]	NS	NS	**+ poor**	+			

+ (bold); independent predictor in multivariable analysis, + (not bold); There was the correlation between mVI and the factor, but it was not an independent predictor,

NS: not significant (there was no correlation.); (blank): Authours didn't mention.

** preoperative tumor size, *** 15μ/L/month

Abbreviations: poor, poorly differentiated type; undiff, undifferentiated type; mode, moderately differentiated type; CM, contiguous multinodular type; IM, intrahepatic metastasis; SNEG, simple nodular type with extranodular growth type; Inf, infiltrative type.

Other issues that should be taken into consideration are as follows:

1. The indication for LT in patients with HCC beyond the Milan criteria. Long-term survival is indeed achievable when MVI is absent; this emphasizes the need to find MVI biomarkers so that LT can be successfully performed for patients with HCC beyond the Milan criteria [50]. The use of the up-to-7 criteria [39] seems, however, to be a reasonable approach [51].
2. The pre-LT treatment. Because of the long waiting time before LT, more patients are being treated preoperatively (locoregional therapy). Furthermore, patients with tumors exceeding the Milan criteria are also being treated so that their HCC can be down-staged [52]. Locoregional tumor therapy has induced >50% tumor necrosis in two-thirds of cases. Even after down-staging, the overall survival and disease-free survival rates are lower than those for patients within the Milan criteria. However, in patients with HCC exceeding the Milan criteria, a complete response after transarterial chemoembolization (according to the amended Response Evaluation Criteria in Solid Tumors guidelines) has been associated with excellent posttransplant outcomes. The complete response was associated with lack of MVI in the expanded liver [53].

CONCLUSIONS

1. MVI is an independent risk factor and/or one of the risk factors for HCC recurrence and poor outcomes after LT. However, a favorable prognosis can be expected when LT is performed for patients with small and well-differentiated HCC because MVI and HCC recurrence are rare in these cases.
2. MVI is most likely to be present in tumors larger than 3 cm, in tumors whose gross pattern is the nodular type with extranodular growth or the confluent multinodular type, and in tumors with a high histological grade (which shows a less differentiated pattern).
3. It is impossible to detect MVI preoperatively by conventional imaging modalities, and there is no widely recognized biomarker for predicting MVI. Because of the significance of MVI with respect to the recurrence of tumors and the prognosis, the possibility of predicting its presence by a comprehensive consideration of tumor characteristics (the size, histological grade, and number) and the potential role of imaging modalities should be further explored; before then, it should be better defined and graded. Further evaluations of the emerging roles of radiological, biological, and molecular profiling of HCC with an MVI signature are needed. Although the use of DCP is not popular in Western countries, its close relationship with the biological behavior of HCC suggests its potential as a biomarker of MVI, and it merits further study.

4. An artificial neural network may become useful for identifying the tumor grade and MVI on the basis of preoperative variables.

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Chapter 3

Microvascular invasion in hepatocellular carcinoma is associated with increased expression of PlGF and VEGFR-1 in the peritumoral tissue

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Abstract

Background and aims: Recent studies showed that microvascular invasion (micro-VI), a frequent phenomenon in hepatocellular carcinoma (HCC), is a strong predictor for tumor recurrence and poor prognosis.

Methods: To potentially establish a profile of micro-VI positive HCC we studied micro-VI in HCC of 43 transplanted patients, from the perspective of a three-step process of vascular invasion: tumor characteristics, epithelial-mesenchymal transition (EMT) and angiogenesis. Gene and protein expression of factors relevant to these 3 processes were investigated: cytokeratin 19 (CK19) and Epithelial cell adhesion molecule (EpCAM) as progenitor cell markers; EMT-associated markers E-cadherin, Twist, Snail, S100A4, Matrix metalloproteinase-9, and angiogenesis markers consisting of Hypoxia inducible factor 1-α, vascular endothelial growth factor (VEGF)-A, Placental growth factor (PlGF), VEGF receptor (VEGFR)-1, VEGFR-2, Angiopoietin (Ang)-1, Ang-2, and Tie-2.

Results: Our results showed that higher tumor grade, decreased tumor E-cadherin expression and a higher expression of PlGF/VEGFR-1 in the adjacent non-cancerous tissue were associated with micro-VI. Progenitor cell characteristics in the tumor were found in a relatively low frequency and there was no correlation with micro-VI.

Conclusions: Our findings show that micro-VI in HCC are influenced not only by tumor characteristics but also by changes in the adjacent non-cancerous tissue.

The higher tumor grade represents more aggressive tumor cells and the more prominent loss of E-cadherin is compatible with higher EMT activity. As PlGF/VEGFR-1 is known to induce pathologic angiogenesis in which abnormal vessels are formed, higher PlGF/VEGFR-1 expression in the adjacent tissue may potentiate vascular invasion. Grading of HCC can contribute to predicting micro-VI and the PlGF/VEGFR-1 expression may provide a possible target of anti-angiogenic treatment to inhibit the generation of abnormal vessels or to normalize the abnormal vessels.

Introduction

Several recent studies have established the pivotal role of microvascular invasion (micro-VI) in hepatocellular carcinoma (HCC) as predictor for poor prognosis and tumor recurrence even after curative resection or liver transplantation [1, 2, 3, 4]. In the so far largest study of 1556 transplanted HCCs from 36 centers it was shown that the presence of micro-VI reduced the 5-year overall survival from 71.2% to 47.4% in the group of patients who fell within the "up-to-7" criteria (HCC with 7 as the sum of the size of the largest tumor in cm and the number of tumor nodules) [3]. In this same group, tumor recurrence at 5 years is 9.1% in the absence of micro-VI but increased to 39.9% when micro-VI is present. The major drawback of establishing micro-VI prior to surgery is the lack of widely recognized and reliable biomarkers [5]. To date, the most reliable identification of micro-VI in HCC can only be performed by histologic examination at the postoperative stage which forms a major obstacle to predict tumor behavior preoperatively and subsequent tailoring of therapy. Apart from the absence of markers for micro-VI, the molecular background of micro-VI in HCC is also poorly understood.

Vascular invasion is a complex process which includes several steps: tumor cell proliferation on the primary site, epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) degradation, cell migration/invasion and tumor angiogenesis [6, 7]. In this study we attempted to establish the cellular and molecular profile of micro-VI positive HCC by investigating vascular invasion from a 3-step perspective: tumor potential, EMT and ECM degradation and angiogenic activity. Tumor characteristics that are known to be associated with micro-VI were documented [1, 8, 9], including size, number of tumor nodules, histologic grade and progenitor cell features. The latter have been reported to be associated with more aggressive tumor behavior and poor prognosis [10, 11]. E-cadherin, Twist, Snail, S100A4 and Matrix metalloproteinase-9 (MMP-9) were included as factors involved in EMT and ECM degradation. Hypoxia inducible factor 1α (HIF-1α) was included because this transcription factor has been reported to induce angiogenesis controlling genes in hypoxic condition and is also engaged in EMT via direct regulation of Twist [12]. Angiogenesis was studied by histologic evaluation of microvessel density (MVD) and gene-expression of the vascular endothelial growth factor (VEGF) family and the Angiopoietin (Ang) system as the 2 best documented angiogenic systems.

Our results demonstrate that micro-VI is associated with tumor characteristics (high-grade tumor), EMT (loss of E-cadherin) and an altered angiogenesis in the peritumoral tissue as indicated by the increased expression of placental growth factor (PIGF) and VEGF receptor (VEGFR)-1.

Patients and methods

Patients and tissue samples

Samples of HCC present in hepatectomy specimens of 43 patients who underwent liver transplantation for end-stage liver disease were included in this study. There were 34 male and 9 female patients with a median age of 56 years (interquartile range; 50-61 years). Gross and microscopic evaluation included tumor size, number of tumors, classification as within or beyond the Milan criteria, and histological grade according to Edmondson and Steiner classification. Micro-VI was defined histopathologically as the presence of cancer cell clusters within a vessel, using hematoxylin-eosin (H&E) staining and CD34 immunostaining.

Gene expression analysis by real time reverse transcription polymerase chain reaction (quantitative RT-PCR)

Fresh-frozen samples of 35 HCC and 25 adjacent non-cancerous tissue samples were available and subjected to RT-PCR assay to quantify the expression levels of messenger RNA (mRNA). An H&E stained slide of all samples was included to check the adequacy of the frozen samples. Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. The extracted RNA was analyzed qualitatively by agarose gel electrophoresis and quantitatively by Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The samples of total RNA were reverse transcribed with Random Hexamers (Promega, Leiden, The Netherlands) and SuperScript III (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Quantitative PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Foster City, CA) using TaqMan PCR assay probe/primers (Assay-on-Demand, Gene Expression Products, Applied Biosystems) for house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), cytokeratin 19 (CK19) (Hs00761767_s1), EpCAM (Hs00158980_m1), E-cadherin (Hs00170423_m1), Twist (Hs00361186_m1), Snail (Hs00195591_m1), S100A4 (Hs00243202), MMP-9 (Hs00234579_m1), HIF-1α (Hs00936366_m1), VEGF-A (Hs00173626_m1), PlGF (Hs00182176_m1), VEGFR-1 (Hs01052936_m1), VEGFR-2 (Hs00176676), Ang-1 (Hs00181613_m1), Ang-2 (Hs00169867_m1), and Tie-2 (Hs00176096_m1). 10 ng complementary DNA was applied for each PCR reaction and all PCR reactions were performed in triplicates. Average cycle threshold (Ct) values per triplicate were calculated for each gene of interest and house keeping gene, yielding ΔCts. To determine the gene expression levels of gene of interest relative to the house keeping gene we next calculated the $2^{-\Delta Ct}$ values. Control samples of distilled water and RNA isolates that were not subjected to reverse transcriptase were consistently found to be negative.

Immunohistochemistry

All primary antibodies used in this study and their dilutions are listed in Table 1. Four-micrometer thick samples were cut from formalin-fixed, paraffin-embedded liver samples which contained both HCC and adjacent non-tumorous tissue. Immunostaining for E-cadherin, CK19, EpCAM and CD34 was performed on the DAKO autostainer (DAKO, Glostrup, Denmark). For Twist, Snail, and S100A4 staining, sections were incubated with the primary antibody (Twist, Snail, S100A4) for one hour at room temperature and stained with the DAKO EnVision + System-HRP Kit (K4009, DAKO) according to the manufacturer's instructions after antigen retrieval. As chromogen 3-amino-9-ethylcarbazole chromogen (DAKO) was applied and hematoxylin (Merck, Darmstadt, Germany) was used as the nuclear counterstain. For VEGF, PlGF and VEGFR-1 staining, frozen sections of 4 µm were incubated overnight at 4°C with the primary antibodies after drying, acetone fixation and blocking of endogenous peroxidase by 30 minutes incubation with 0.3% H_2O_2 at room temperature. The subsequent steps were similar to those described above.

Table 1. Primary antibodies used in immunohistochemistry

Antibody	Dilution	Company	Catalog No.
Mouse monoclonal anti-E-cadherin [36]	1:4000	BD Transduction Laboratories	C20820
Mouse monoclonal anti-CK19 [RCK108]	1:100	DakoCytomation	M0888
Mouse monoclonal anti-EpCAM [Ber-EP4]	1:100	DakoCytomation	M0804
Mouse monoclonal anti-CD34 [QBEnd-10]	1:20	DakoCytomation	M7165
Rabbit polyclonal anti-Twist	1:300	Abcam	ab50581
Rabbit polyclonal anti-Snail+Slug	1:500	Abcam	ab85931
Rabbit polyclonal anti-S100A4	1:200	DakoCytomation	A5114
Rabbit polyclonal anti-VEGF-A (A-20)	1:100	Santa Cruz	sc-152
Rabbit polyclonal anti-PlGF	1:100	Abcam	ab9542
Rabbit polyclonal anti-VEGFR-1	1:100	Abcam	ab2350

All primary antibodies were diluted in 1% bovine serum albumin/phosphate- buffered saline.

Evaluation of immunoreactivity

Semiquantitative assessment of E-cadherin expression was performed according to the method of Asayama et al. in which staining is graded as: 'preserved' if more, and 'reduced' if less than 90% of tumor cells showed a membranous expression [13]. For CK19 and EpCAM, if more than 5 % of tumor cells showed membranous

expression it was considered positive [11]. For Twist and S100A4, the staining was considered positive if more than 10% and 30% of tumor cells respectively showed nuclear expression [14, 15]. The evaluation was performed by two liver pathologists (ASHG and HK) blinded to the patients' clinicopathological data.

Determination of MVD

MVD was assessed in HCC tissues of which paraffin embedded sections were stained with anti-CD34 antibody and scanned on NanoZoomer 2.0-HT (Hamamatsu Photonics, Japan). The observation and the area measurement were done using NDP viewer software (Hamamatsu Photonics). First, five areas of $0.74mm^2$ with the most intense staining (hot spots) were selected. Regardless of the vessel lumen structure, every stained cell in the hot spot was counted as one microvessel. In the case that a positive staining appears to be linear and to outline nests of tumor cells, we used a modified counting method introduced by Tanigawa et al, in which the length of a microvessel of 40 micrometer was calculated as one point [16]. The average of 5 areas counts expressed as the absolute number of microvessels per $0.74mm^2$ was taken as the MVD.

Statistical analysis

Quantitative data were expressed as median and interquartile range. A two-tailed nonparametric test was used for comparison of groups; either the Mann-Whitney U test for non-related samples (comparison between micro-VI negative and positive group) or the Wilcoxon signed-ranks test for related samples (comparison between HCC and adjacent non-tumorous tissue). Categorical variables were compared using Fisher's exact test. P values of less than 0.05 were considered statistically significant. All statistical analysis was performed using PASW Statistics 18 software (SPSS, Chicago, IL, USA).

Results

Tumor characteristics: higher tumor grade is associated with micro-VI

From a total of 43 cases, 30 cases (70%) showed micro-VI. The median of the largest tumor size and the number of tumors were 2.6 cm (interquartile range: 2-4.1 cm) and 2 (interquartile range: 1-5) respectively. There was a significant difference in tumor grade between the micro-VI positive and micro-VI negative groups (P=0.039). Nine cases were high grade tumors (Edmondson III-IV), and all these cases showed micro-VI. Among 34 low grade cases (Edmondson I- II), 21 cases (62%) showed micro-VI. Comparisons of patient and tumor characteristics according to the presence/absence of micro-VI are shown in Table 2. There was no

statistical difference between the absence and presence of micro-VI based on age, gender, tumor size, number of tumor nodules, postoperative status of Milan criteria, and microvessel density. In our series, 2/43 HCCs contained CK19-positive cells (4.6%) and 6/43 EpCAM-positive cells (18%). In the micro-VI negative group (13/43) there were 2 EpCAM-positive cases and no CK19-positive case, whereas in the micro-VI positive group (30/43) there were 4 EpCAM-positive cases and 2 CK19-positive cases. No correlation was found between micro-VI and the cases expressing these progenitor markers.

Table 2. Patient and tumor characteristics

	micro-VI negative n = 13	micro-VI positive n= 30	P value
Age, median (range)	59 (48-66 yrs)	54 (50-60 yrs)	0.222
Male/female	11/2	23/7	0.699
HCV negative / positive	10/3	22/8	1
HBV negativ / positive	13/0	28/2	1
Tumor size <5cm / >5cm	12/1	20/10	0.129
Number of tumors; single / multiple	4/9	13/17	0.513
Milan criteria; within / beyond	8/5	16/14	0.743
Edmondson grade; I and II / III and IV	13/0	21/9	0.039
CK19 negative / positive	13/0	28/2	1
EpCAM negative / positive	11/2	26/4	1
CD34-MVD	311 ± 74	290 ± 99	0.472

Gene and protein expression studies

The results of the quantitative RT-PCR analysis are summarized in **Fig. 1** and **2**. **Fig. 1** demonstrates the comparison of gene expression levels between the micro-VI negative and positive group, and **Fig. 2** demonstrates the comparison of gene expression levels between HCC and its adjacent non-cancerous tissue within each group.

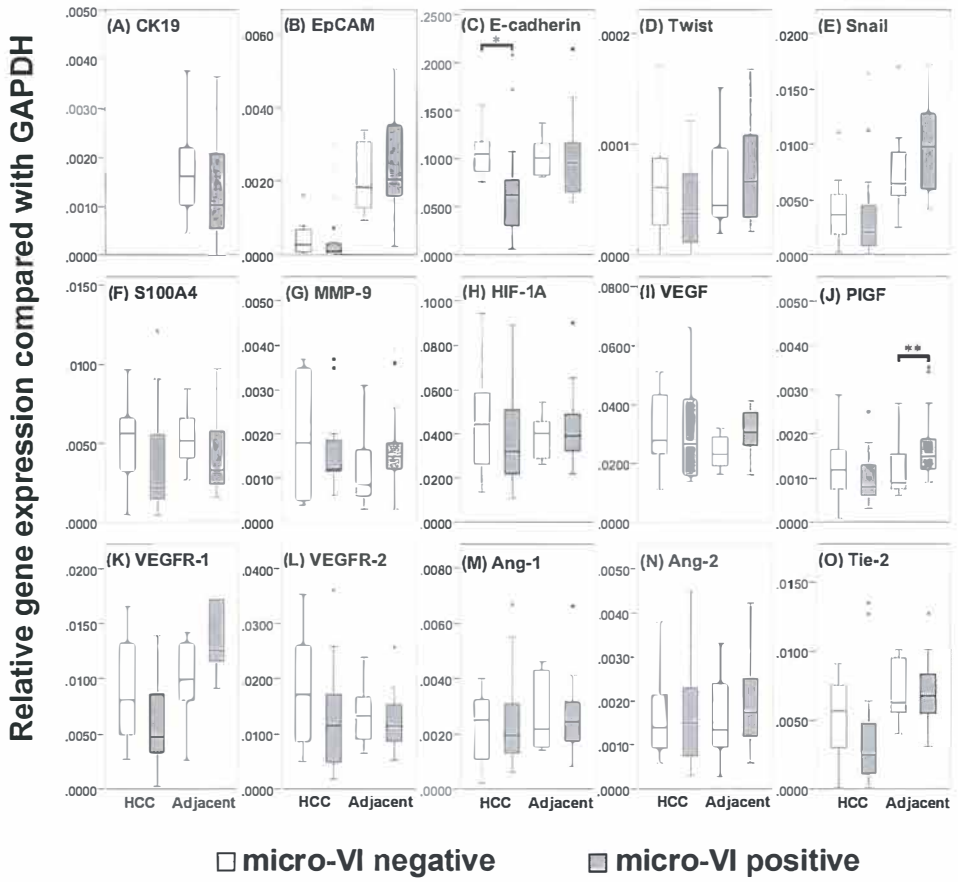

Figure 1. **Comparison of gene expression levels between microvascular invasion (micro-VI) negative and positive group in HCC tissue (negative; n=11, positive; n=24) or adjacent noncancerous tissue (negative; n=8, positive; n=17).**
Box-plot graphs showing relative gene expression for CK19 (A), EpCAM (B), E-cadherin (C), Twist (D), Snail (E), S100A4 (F), MMP-9(G), HIF-1A (H), VEGF (I), PIGF (J), VEGFR-1 (K), VEGFR-2 (L), Ang-1 (M), Ang-2 (N), Tie-2(O). The box and the horizontal line inside the box represent the interquartile (25-75%) range and the median value respectively. Capped bars indicate the 5th and 95th percentiles and small dots the outliers. *P<0.001, **P<0.05

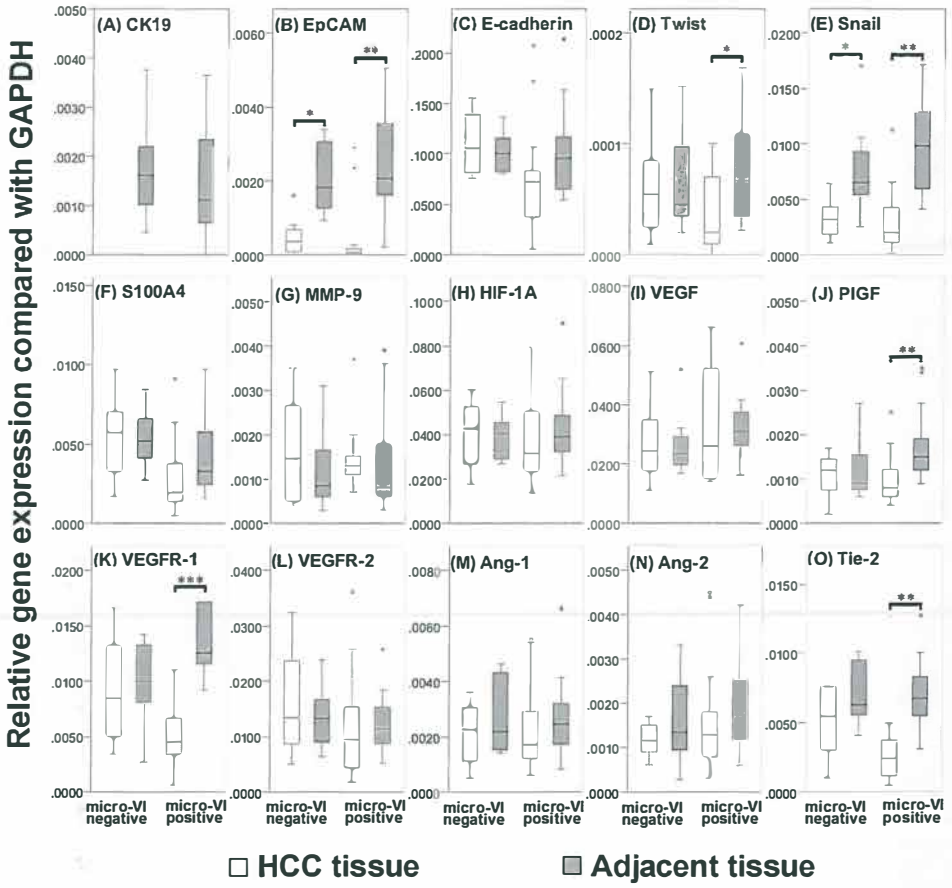

Figure 2. **Comparison of gene expression levels between HCC and adjacent noncancerous tissue in microvascular invasion (micro-VI) negative group (8 paired) or micro-VI positive group (17 paired).**
Box-plot graphs showing relative gene expression for CK19 (A), EpCAM (B), E-cadherin (C), Twist (D), Snail (E), S100A4 (F), MMP-9(G), HIF-1A (H), VEGF (I), PlGF (J), VEGFR-1 (K), VEGFR-2 (L), Ang-1 (M), Ang-2 (N), Tie-2(O). The box and the horizontal line inside the box represent the interquartile (25-75%) range and the median value respectively. Capped bars indicate the 5th and 95th percentiles and small dots the outliers. *P<0.05, **P<0.01, ***P<0.001

Progenitor cell characteristics: no differences between micro-VI positive and negative groups

Since the CK19 mRNA was not amplified in most HCC tissues in the qRT-PCR study, we excluded it from **Fig. 1(A) and 2(A)**. In contrast EpCAM mRNA was properly amplified in both HCC and adjacent tissue. Micro-VI negative and positive tumors as well as the adjacent tissue of both groups did not show significant differences regarding EpCAM expression (**Fig. 1(B) and 2(B)**). However, there was a significantly lower gene expression level of EpCAM in the tumor compared with

its adjacent tissue in both the micro-VI negative and positive groups, and this difference was more prominent in the micro-VI positive group.

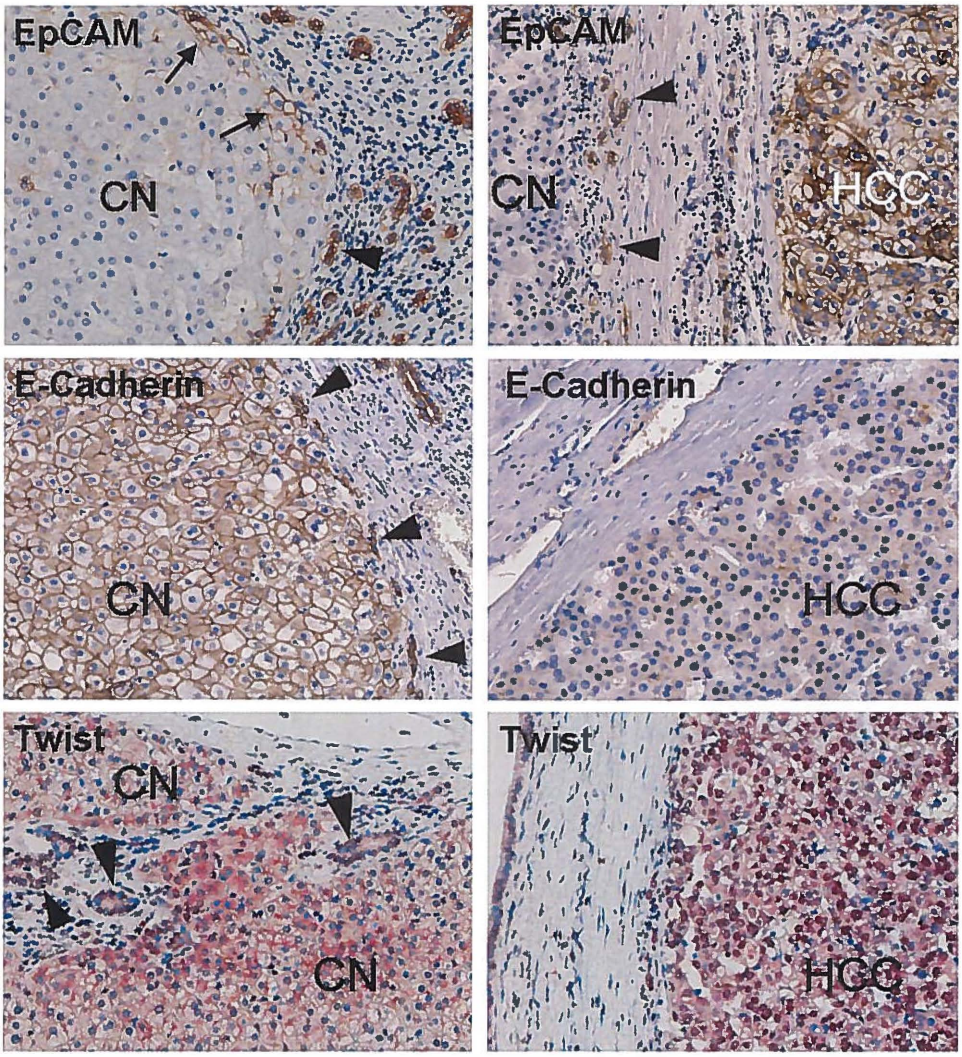

Figure 3. **Immunohistochemistry for EpCAM, E-cadherin and Twist in adjacent non-tumorous tissue (left panel) and HCC (right panel).**
EpCAM: *Left panel*: some non-tumorous hepatocytes at the periphery of a cirrhotic nodule are positive for EpCAM (arrows), as are all bile ductules (arrow heads). *Right panel:* example of an EpCAM positive HCC and EpCAM negative hepatocytes in the neighbouring cirrhotic nodule (CN) Bile ductules surrounding the cirrhotic nodule are EpCAM positive (arrow heads)
E-cadherin: *left panel*: non-tumorous hepatocytes and surrounding ductules (arrow heads) are positive for E-cadherin. *Right panel*: loss of E-cadherin expression on tumor cells.
Twist: *left panel*: nuclear and cytoplasmic expression of Twist in ductules (arrow heads) surrounding cirrhotic nodule (CN) in which the hepatocytes show cytoplasmic but no nuclear Twist expression. *Right panel:* diffuse nuclear and cytoplasmic expression of Twist in HCC.

Immunohistological distribution of CK19 and EpCAM expressing HCC was mentioned above as part of the tumor characteristics. In the adjacent tissue, CK19 and EpCAM were predominantly expressed by cholangiocytes of biliary ductules. These ductules were readily found in areas adjacent to non-cancerous cirrhotic nodules but were scarce or absent at the margins of HCC nodules (**Fig. 3**). Some cases also showed small rims of EpCAM positive hepatocytes in the non-cancerous cirrhotic nodules while there were no cases showing CK19 positive hepatocytes.

EMT related factors: decreased E-cadherin in micro-VI positive HCC Micro-VI positive HCC showed lower E-cadherin mRNA expression than micro-VI negative HCC ($P<0.001$) but between HCC and their adjacent non-cancerous counterparts there was no significant difference. The adjacent non-cancerous tissue of the micro-VI positive and negative groups did not show differences in E-cadherin levels either. The gene expression level of Twist was significantly lower in HCC compared to its adjacent tissue in the micro-VI positive group. Compared to the adjacent non-cancerous tissue Snail expression in HCC was significantly lower both in the micro-VI positive and micro-VI negative groups. Comparisons between micro-VI negative and microVI-positive groups revealed no significant differences in the gene expression levels of Twist, Snail, and S100A4.

The results of the immunohistochemistry are summarized in Table 3. In the micro-VI negative group, 2/13 cases showed reduced E-cadherin expression, whereas in the micro-VI positive group, 15/30 cases showed this decrease including complete loss of E-cadherin in one case. The reduced protein expression of E-cadherin was correlated with micro-VI ($P=0.045$). In the neighboring non-cancerous tissue E-cadherin expression was present on hepatocytes and ductular cells (**Fig. 3**). Immunohistologically, most tumor cells showed cytoplasmic expression of Twist, and 10 cases showed positive nuclear expression of Twist including one case with diffuse nuclear expression. In addition, we observed ductular cells around cirrhotic nodules in adjacent non-cancerous tissue that also showed nuclear expression of Twist (**Fig. 3**). None of the cases showed expression of S100A4 in tumor cells while the vascular wall of the unpaired arteries in HCC and stromal cells, inflammatory cells and Kupffer cells were positive for S100A4. Of note, ductular cells in the adjacent non-cancerous tissue also showed positive cytoplasmic expression for S100A4.

Table 3. Comparison of the results of immunohistochemistry for EMT markers dependent on the existence of micro-VI

	micro-VI negative	micro-VI positive	P value
E-cadherin preserved/reduced or absent on tumor hepatocytes	11/2	15/15	0.045
Twist negative/positive on tumorhepatocytes	10/3	21/9	0.727
Twist negative/positive on ductules in NT part	7/6	11/19	0.332
Snail negative/positive on tumor hepatocytes	11/2	28/2	0.572
Snail negative/positive on ductules in NT part	11/2	25/5	1
S100A4 negative/positive on tumor hepatocytes	13/0	30/0	1

NT, non-tumorous

Angiogenesis: the adjacent tissue of the micro-VI positive group showed increased expression of PIGF/VEGFR-1

Apart from PIGF, mRNA expression levels of all studied angiogenesis related factors showed no differences between the micro-VI positive and micro-VI negative groups. The expression level of PIGF mRNA in the adjacent tissue of the micro-VI positive group was significantly higher compared to that of the adjacent tissue of the micro-VI negative group (P=0.021) (**Fig. 1(J)**). A similar trend was found for VEGFR-1 although the difference was of statistical borderline significance (P= 0.057) (**Fig. 1(K)**). When we compared HCC with its adjacent tissue, the expression of PIGF, VEGFR-1, and Tie-2 were significantly higher in the adjacent tissue in the micro-VI positive group (**Fig. 2(J), (K), and (O)**) which was not the case in the micro-VI negative group.

Immunohistologically VEGF, PIGF, and VEGFR-1 were present on vascular and sinusoidal endothelial cells in HCC and non-cancerous adjacent tissue in both the micro-VI positive and negative groups (**Fig. 4**).

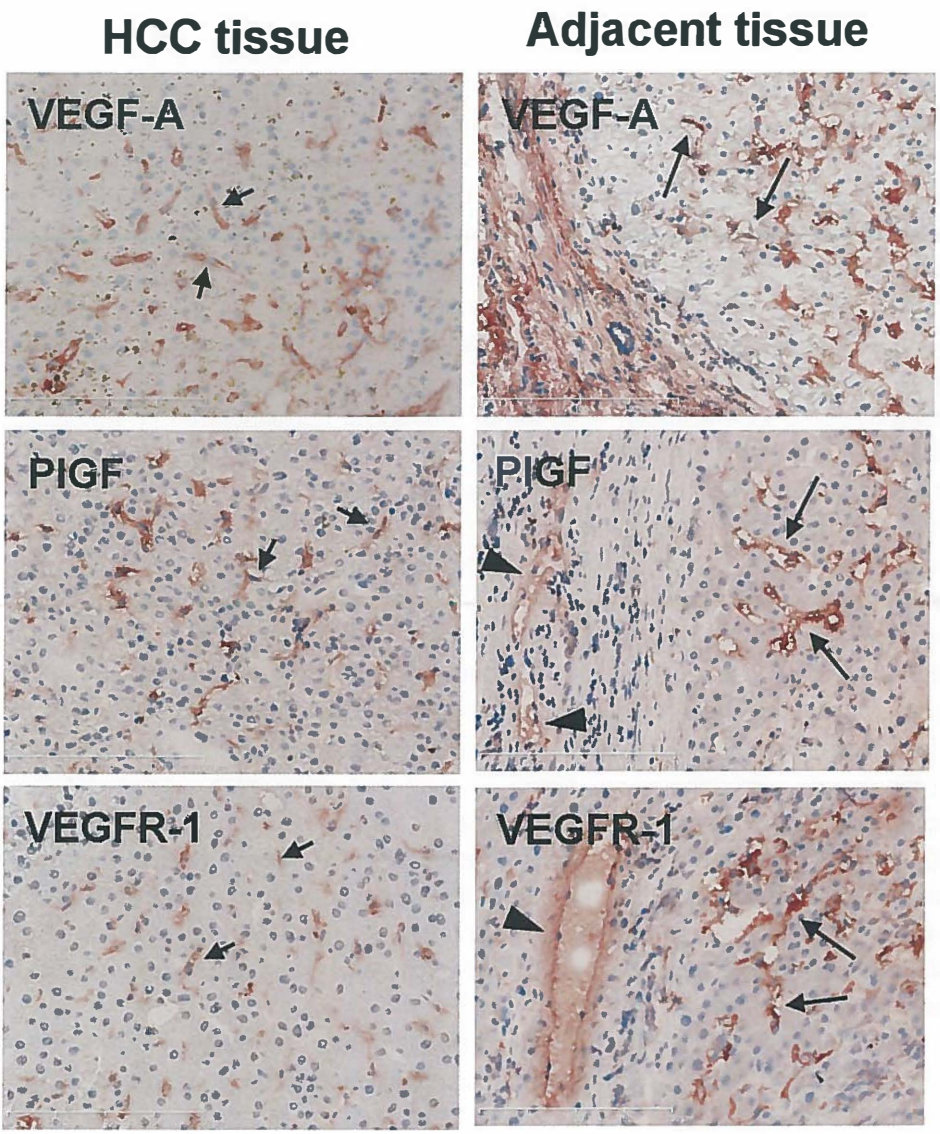

Figure 4. **Immunohistochemistry for VEGF, PIGF, and VEGFR-1 in HCC and adjacent tissue.** Immunohistochemistry showing expression of VEGF, PIGF and VEGFR-1 in tumor endothelial cells (short arrows) and vascular and sinusoidal endothelial cells (arrowheads and long arrows) in the adjacent liver .

Discussion

In this study, we investigated the profile of micro-VI in HCC from a 3 levels-perspective: tumor potential, EMT related factors, and angiogenic potential. Our results showed that micro-VI positive HCC is characterized by a high-grade tumor and decreased E-cadherin expression by tumor cells while the adjacent non-cancerous compartment contains an increased expression of the angiogenic factors PlGF and VEGFR-1.

Of the several tumor characteristics that were assessed, a higher tumor grade was the only feature that significantly distinguished the micro-VI positive HCC from the micro-VI negative one. A number of other studies reported variable correlations between micro-VI with tumor size, tumor grade and number of tumor nodules [5]. These inconsistencies may be influenced by the fact that HCC nodules within a cirrhotic liver represent both multifocal malignant transformation of cirrhotic nodules and intra organ metastases. These 2 types of nodules which are distinguishable by molecular analysis only and not by routine histology, represent different modes of tumor growth, i.e., oligoclonal expansion and metastatic growth [19, 20]. Intra organ metastatic nodules have been reported to be associated with more aggressive growth compared to multicentric tumor nodules, leading to portal-vein invasion, higher incidence of tumor recurrence and poor survival [20]. Furthermore, differences in sample size of the studies, variable definitions of micro-VI and protocols of histopathological tumor examination may also contribute to the inconsistent correlations. Nevertheless, a correlation of micro-VI and high-grade HCC within size-and-number group categories has been established in the Metroticket study which so far represents the largest cohort of HCC ever published, comprising 1556 patients from 36 centers [3]. High grade tumors are less differentiated tumors with more aggressive behavior, i.e., exhibiting more invasive potential.

Tumor aggressiveness is amplified by the presence of cancer cells with stem cells signatures (CSS), a phenomenon that has also been observed in HCC in which CSS is defined by the expression of e.g., CK19, EpCAM, CD133 [10, 21, 22]. A recent study reported an association between macrovascular invasion and hematogenous metastasis in the presence of a subpopulation of CSS defined as $CD133+/CD44^{high}$ tumor cells in HCC [23]. Another study showed a correlation of both macro-VI and micro-VI with HCC containing CK19 positive cells in which 18% of the cases contained CK19 positive CSS [10]. Both on the gene and protein expression levels of CK19 and EpCAM, we found a lower frequency of CSS containing tumors than others and no correlation between CSS containing HCC with micro-VI. A difference in etiology of the underlying liver diseases may influence these discrepancies. Both hepatitis B virus (HBV) and hepatitis C virus

(HCV) have been reported to induce CSS in HCC [24, 25] but only 30% of our cases were HCV or HBV related in contrast with 85% of the cases in the series of Kim et al [10].

In the micro-VI positive group we found a decrease of gene and protein expression of E-cadherin by HCC cells which is regarded as a manifestation of EMT and an important initial step in the tumor invasion process due to the loss of cell-cell adherence [26]. The other studied EMT related factors did not distinguish between micro-VI positive and negative groups. The low expression of Snail and Twist in the tumor matches the low expression of CSS features in our HCC as expression of these EMT transcription factors is associated with CSS properties [10, 27]. Accordingly, nuclear protein expression of Snail and Twist in tumor cells was also found in a few cases only.

The potential importance of changes in peritumoral tissue in the pathogenesis of micro-VI is illustrated by the finding of a higher expression of PlGF and VEGFR-1 in the non-cancerous adjacent tissue of micro-VI positive HCC compared to the expression levels in the tumor. Results of several studies in tumor models have established the central role of the PlGF/VEGFR-1 signaling in tumor angiogenesis. PlGF stimulates endothelial cell growth, survival and migration in pathological conditions but appears to be redundant in normal vascular development and maintenance [28, 29]. Under pathological conditions PlGF potentiates the angiogenic response to VEGF by displacement of VEGF from VEGFR-1 to VEGFR-2 and by activating VEGFR-1. The synergy of VEGF mediated VEGFR-2 activation and PlGF mediated VEGFR-1 activation leads to amplification of the angiogenic response [28]. Overexpression of PlGF stimulates tumor growth due to increased tumor vascularization consisting of abnormal blood vessels with incomplete coverage by pericytes and an irregular basement membrane [30]. Conversely, blockage of PlGF inhibits tumor vessel arterialization and abnormalization and also impedes tumor growth in experimental HCC without significant effect in vessel density [31]. Based on these tumor-angiogenic characteristics it is conceivable that the increased PlGF/VEGFR-1 expression in the adjacent tissue could stimulate generation of abnormal vessels that are permissive to invasion due to its abnormal architecture. Whether such a process truly exists in micro-VI positive HCC and may be inhibited by anti-PlGF would need further investigation.

In summary, we found that the profile of micro-VI positive HCC consists of high grade HCC and loss of tumor E-cadherin while the adjacent non-cancerous tissue showed increased expression of PlGF and VEGFR-1. The grading of HCC might contribute in predicting the presence of micro-VI along with other diagnostic tools, e.g., imaging studies. The PlGF/VEGFR-1 activity may provide a target to

anti-angiogenic therapy to inhibit abnormal vessel formation and decrease subsequent potentiation of vascular invasion.

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Chapter 4

Decreased numbers of biliary ductules in peritumoral fibrous septa is associated with microvascular invasion in Hepatocellular Carcinoma

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Abstract

Microvascular invasion (micro-VI) is a frequent finding and strong prognostic marker in patients with hepatocellular carcinoma (HCC) for which there is no reliable predictive marker before surgery. Due to its microscopic nature the diagnosis is based on histopathological examination of the resected tumor. In a previous study we found evidence that micro-VI is associated with alterations in the angiogenic status of the peritumoral area. To further investigate the association between micro-VI and peritumoral changes we focused on the peritumoral area and investigated the changes herein in comparison with stromal areas distant from the tumor.

We isolated the peritumoral fibrous septa (PT septa) and fibrous septa distant to the tumor (D septa) using laser microdissection technique in 11 HCC livers and measured the gene expression levels of factors possibly related with micro-VI. Those were growth factors related with angiogenesis, epithelial mesenchymal transition and factors characterizing hepatic progenitor cells.

We found decreased gene expression levels of epithelial cell adhesion molecule (EpCAM) and E-cadherin within PT septa relative to its corresponding D septa in the micro-VI positive HCCs, not in micro-VI negative HCCs. A parallel finding on the protein level was manifested by decreased numbers of EpCAM positive ductules in the PT septa. The association of micro-VI with significantly lower numbers of ductules in the PT septa compared with its D-septa was confirmed by a validation study in a second cohort of 25 patients.

Conclusion: Our findings confirm that several types of changes in peritumoral areas may play a role in micro-VI in HCC which corroborate the notion that the tumor microenvironment can contribute to cancer growth and progression. From a diagnostic practical perspective our finding may also be a potential predictive tool for micro-VI.

Introduction

It is well known that macrovascular invasion in hepatocellular carcinoma (HCC), which can be diagnosed before surgery by conventional imaging modalities, is a poor prognostic factor and a contraindication to surgical treatments [1]. Microvascular invasion (micro-VI), which is a frequent phenomenon in HCC is also an independent prognostic factor [2, 3] associated with tumor recurrence and poor survival [4]. In contrast with macrovascular invasion, the presence of micro-VI can only be identified by histopathological examination of the resected specimen due to its microscopic nature and the lack of reliable predictive markers before surgery [5]. Recently Kim et al showed that peritumoral hypointensity seen on the hepatobiliary phase of gadoxetate disodium-enhanced magnetic resonance imaging is a highly specific radiological marker predictive of micro-VI although with insufficient sensitivity of 38.3% [6]. Paraoxonase 1A, identified by proteomic analysis, is recently reported as a promising serum biochemical predictor for micro-VI with moderate accuracy [7]. Although recent progress on imaging modalities and proteome analysis is uncovering relevant markers for micro-VI, to date there is still no reliable predictive marker with sufficiently high sensitivity and specificity.

In a previous study we documented that a HCC containing micro-VI is characterized by high grade tumor, significant loss of E-cadherin by tumor cells and increased gene and protein expression of VEGFR1 and PIGF in the adjacent non-cancerous tissue [8]. The latter finding of abnormal angiogenic status in the tumor surrounding tissue is compatible with recent insights that cancer stromal tissue is not a passive bystander, but is actively involved in cancer progression and invasion of cancer cells [9, 10].

To further investigate the changes in tumor stroma associated with micro-VI we designed the present study in which we focused on the fibrous septa adjacent to HCC, the peritumoral (PT) septum and fibrous septa surrounding cirrhotic nodules distant from the tumor (D) present in the same liver. The septa were isolated by laser microdissection (LMD) and we studied gene and protein expression of factors involved in angiogenesis and epithelial mesenchymal transition (EMT). Factors related to liver progenitor cell characteristics, epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (K19) were also included as the septa also contain biliary ductules which harbor these progenitor cells. Following the identification of the genes that distinguish micro-VI positive from micro-VI negative HCC, the expression of the related proteins were studied in a second cohort of 25 HCC samples to validate the initial findings.

Patients and Methods

Patients and tissue samples

Archival liver tissue samples were obtained from patients who underwent liver transplantation for cirrhosis associated HCC at the University Medical Center Groningen. The largest tumor diameter, number of tumor-nodules, histological grade (according to Edmondson and Steiner classification), and the presence of micro-VI were evaluated on the available paraffin sections.

Eleven cases of which frozen tissue samples were available containing both PT septa and D-septa were included as the first cohort of the LMD study and subsequent gene expression analysis.

The validation study consisted of a second cohort of 25 HCC samples subjected to immunohistochemical study. Two paraffin sections of each case were used in this study, one containing a PT-septum and another one of the corresponding cirrhotic liver, sampled at least 3 cm distant from the tumor, for the D-septum.

Laser microdissection

Fresh frozen tissues were taken from PT-stroma and a sample of the D-septum located at least 3cm distant from the tumor. Nine-µm cryosections mounted on polyethylene terephthalate membrane frame slides (Leica Microsystems, Wetzlar, Germany) were fixed in cold acetone for 2 minutes, stained with Mayer's hematoxylin for 1 minute, washed with diethyl pyrocarbonate-treated water, and air-dried. LMD was performed using a Leica LMD6500 microdissection system (Leica Microsystems). Tumor cells and hepatocytes were excluded carefully. Figure 1 shows representative pictures before and after LMD. The average surface area of the microdissected area in each case was 9 mm^2.

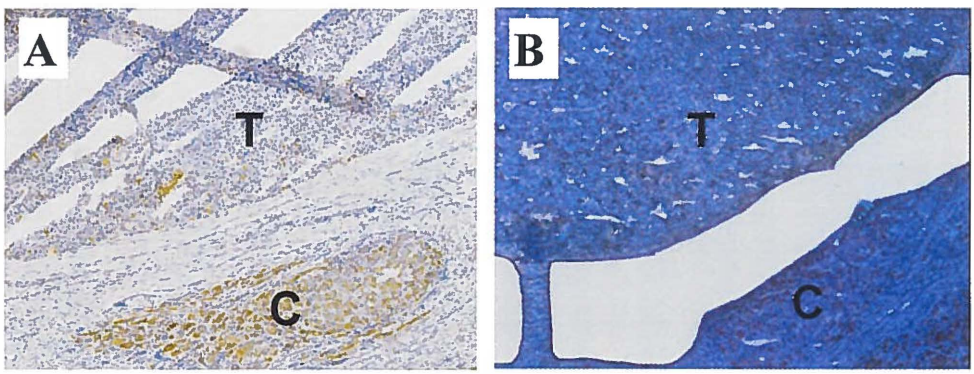

Figure 1. Photomicrograph of a peritumoral septum before (A) and after (B) microdissection.
T: tumor. C: cirrhotic nodule.

Gene expression analysis by quantitative real-time reverse transcription polymerase chain reaction

Total RNA was isolated from these microdissected samples by using RNeasy Plus Micro Kit (Qiagen, Westburg, Leusden, the Netherlands) according to the manufacturer's instructions. The samples of total RNA were reverse transcribed with Random Hexamers (Promega, Leiden, the Netherlands) and SuperScript III (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Foster City, CA) using TaqMan PCR assay probe/primers (Assay-on-Demand, Gene Expression Products, Applied Biosystems) for house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), K19 (Hs00761767_s1), EpCAM (Hs00158980_m1), E-cadherin (Hs00170423_m1), N-cadherin (Hs00983056_m1), Snail (Hs00195591_m1), S100A4 (Hs00243202), MMP-2 (Hs01548727_m1), MMP-9 (Hs00234579_m1), HIF-1α (Hs00936366_m1), PIGF (Hs00182176_m1), VEGF-A (Hs00173626_m1), VEGFR-1 (Hs01052936_m1), VEGFR-2 (Hs00176676), Ang-1 (Hs00181613_m1), Ang-2 (Hs00169867_m1), Tie-2 (Hs00176096_m1), and CD34 (Hs00990732_m1). Duplicate real-time RT-PCR analyses were executed for each sample, and the threshold cycle values (Ct) were analyzed by using Sequence Detector Systems version 2.4 software (Applied Biosystems). Gene expression was normalized to the expression of the housekeeping gene GAPDH, and relative mRNA level was calculated by $2^{-\Delta Ct}$ method. Control samples of distilled water were consistently found to be negative.

Immunohistochemistry

Four µm paraffin sections were deparaffinized in xylene and graded ethanol. After enzymatic digestion with 0.1 % proteinase (CAS Number 9014-01-1, Sigma–Aldrych, Zwijndrecht, the Netherlands) for 15 minutes and endogenous peroxidase blocking with 0.3 % H2O2 for 30 minutes, slides were incubated with anti-EpCAM mouse monoclonal antibody (dilution; 1:1500, clone VU-1D9, Cat. No. OP187-100UG, Merck Chemicals, Darmstadt, Germany) overnight at 4°c and stained with the DAKO EnVision + System-HRP (Cat. No. K4006, DAKO, Glostrup, Denmark) according to the manufacturer's instructions. After the color development with 3,3'-Diaminobenzidine, hematoxylin was used as a nuclear counterstain.

Evaluation of EpCAM staining

A relative comparison of the number of EpCAM-positive ductules in fibrous septa was done between the PT and D-septa for each individual case. Representative septal areas of similar width were selected, photographed and compared with each

other on screen. The results were categorized into three groups with regard to the contents of EpCAM positive ductules: PT>D (PT area contains at least more than 10% of ductules than the distant area) 10%); PT=D (the difference in number of ductules does not exceed 10%); and PT<D (PT septum contains at least 10% less ductules than the D septum). The evaluation was performed by two liver pathologists (ASHG and HK) blinded to the patients' clinicopathological data.

Statistical analysis

In the gene expression study, a two-tailed nonparametric test was used for comparison of groups; either the Mann-Whitney U test for non-related samples (comparison between micro-VI negative and positive group) or the Wilcoxon signed-ranks test for related samples (comparison between close and distant area). In the immunohistochemical study, categorical variables were compared using Fisher's exact test (2x3 crosstabs analysis). Two tailed P values of less than 0.05 were considered statistically significant. All statistical analysis was performed using PASW Statistics 18 software (SPSS, Chicago, IL, USA).

Results

Gene expression analysis of the first cohort

The results of the gene expression analysis were summarized in figures 2 and 3. When we compared PT with D-septa within each of the micro-VI groups, only in the micro-VI positive group there was a significant difference with regard to EpCAM and E-cadherin expression. PT septa showed a lower expression than D septa for both EpCAM (p= 0.03) and E-cadherin (p=0.02) (red rectangle in figure 2). All angiogenic and other EMT related factors did not show significant differences.

Comparison between the micro-VI positive with the micro-VI negative groups did not show differences in gene expression levels for all tested factors, both in PT and D septa (Fig.3).

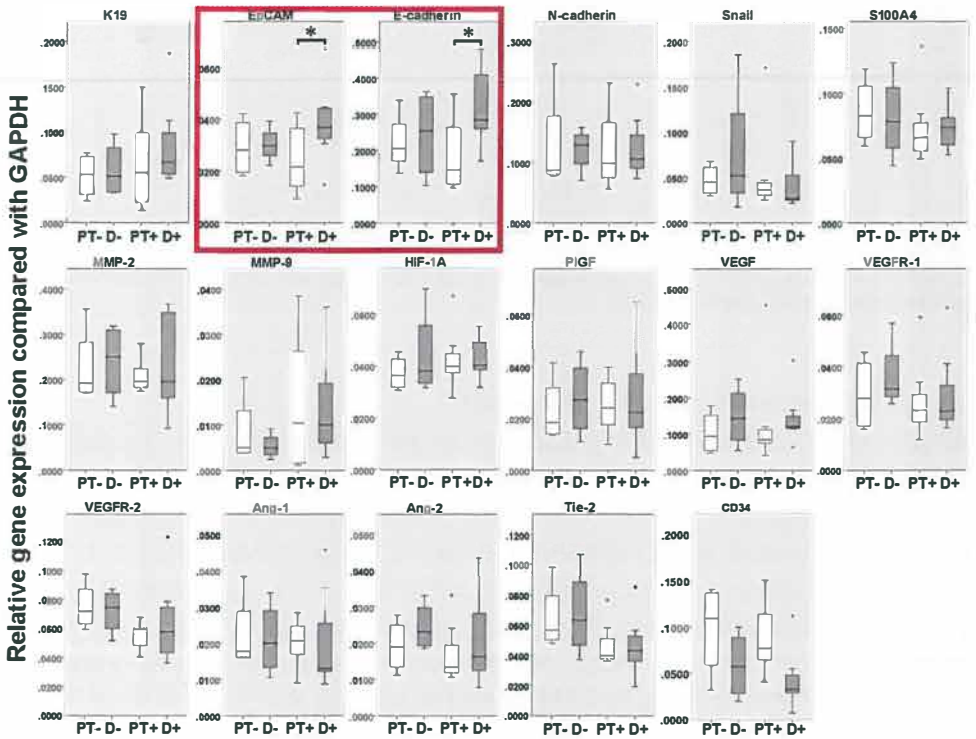

Figure 2. Comparison of gene expression levels between peritumoral septa (PT) and distant septa (D) in microvascular invasion negative group (PT- and D-) or microvascular invasion positive group (PT+ and D+). Boxes represent the interquartile (25-75%) range and the median values. Capped bars indicate the 5th and 95th percentiles and small dots the outliers. *= p<0.05.

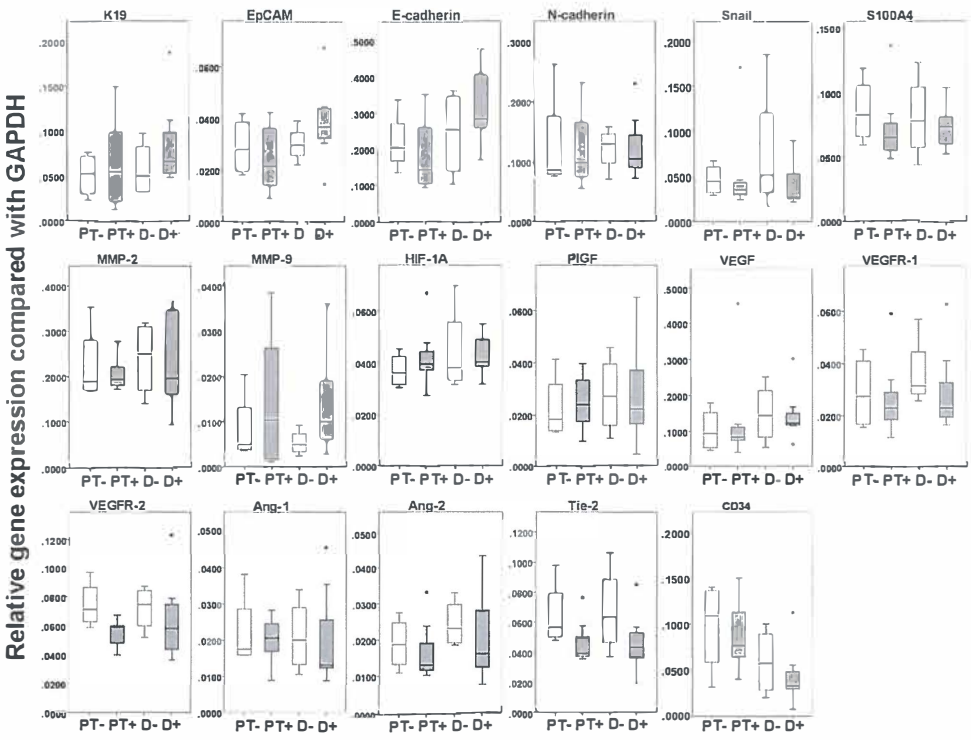

Figure 3. Comparison of gene expression levels between microvascular invasion negative and positive groups in peritumoral septa (PT- and PT+) or septa distant to primary tumor (D- and D+). Boxes represent the interquartile (25-75%) range and the median value. Capped bars indicate the 5th and 95th percentiles and small dots the outliers.

Immunohistochemistry in the first cohort

EpCAM and E-cadherin staining were performed as these were the 2 proteins that showed a significant difference in gene expression level between the PT and D septa in the micro-VI positive HCC. Both proteins were diffusely expressed by biliary ductular cells which represent the only epithelial cell component in the PT and D septa along with ductal cells of a few bile ducts. No hepatocytes were present in the septa studied. To further evaluate the background of differences between the PT and D septa, the number of ductules in both septa was comparatively studied using EpCAM stained slides (Fig. 4).

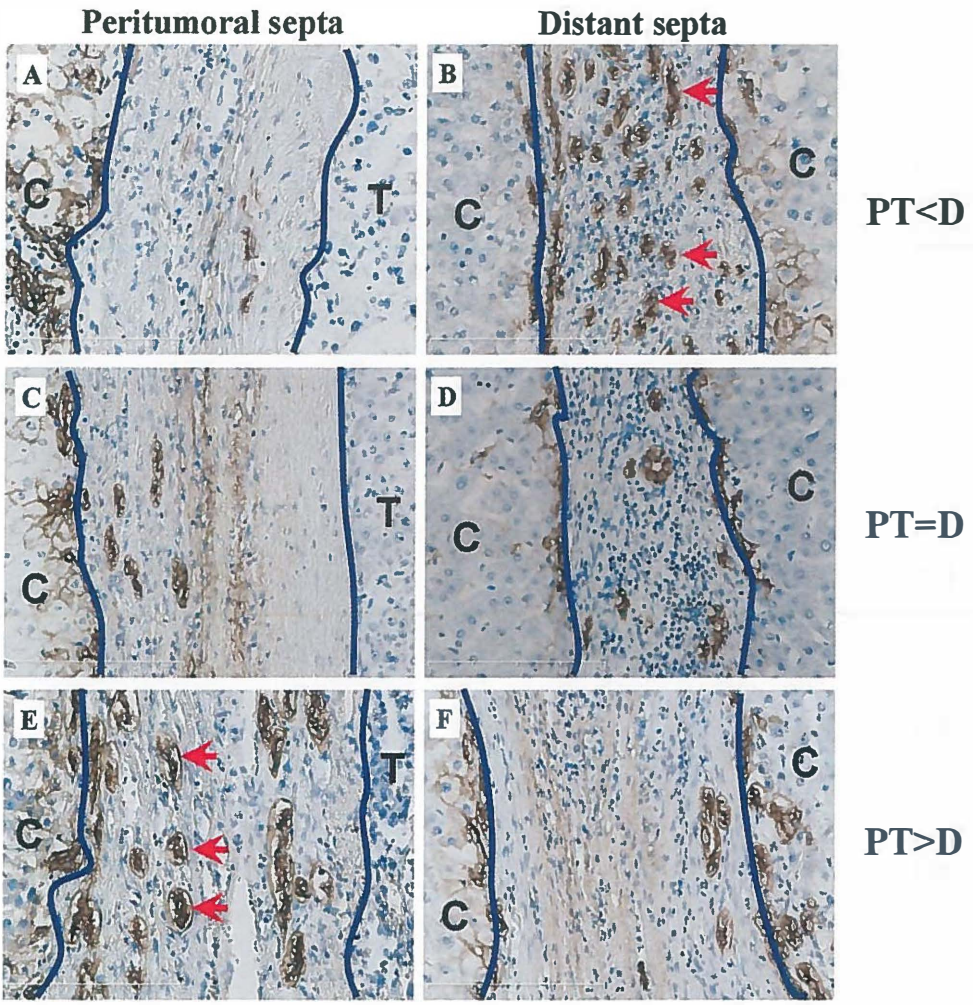

Figure 4. Representative pictures of EpCAM staining in fibrous septa. Two pictures next to each other (A and B, C and D, or E and F) were taken from the same liver. The relative comparison of the number of EpCAM positive ductules between the two septa was classified into PT<D, PT=D, and PT>D, respectively. Blue lines indicate the rim between tumor (T) or cirrhotic (C) nodule and fibrous septum. Arrowheads: EpCAM expressing ductules. Red arrowheads: ductules.

Analysis of the number of ductules in PT and D septa showed similar numbers of ductules in 5 out of 11 cases (45%). In another 5 cases (45%), the PT septa showed lower numbers of ductules and all these 5 cases were micro-VI positive. One out of 11 micro-VI negative case showed more ductules in the PT septa (22%). Although these results were not statistically significant there was a similar trend with the gene expression levels of EpCAM. Further analysis to validate these initial findings was carried out in the second, larger cohort of 25 HCC samples.

Immunohistochemistry of the second validation cohort

Results were summarized in figure 5. As shown, the number of ductules in PT and D septa was similar in 10 out of 25 cases (40%). In 8 out of 25 cases (32%), the PT area showed lower numbers of ductules, and all these 8 cases were micro-VI positive HCC. On the other hand, the PT septa showed more ductules in 7 out of 25 cases (28%), and 6 out of 7 were micro-VI negative HCC. In this larger, validation cohort, a significant relationship between micro-VI status and the number of ductules was identified (P=0.003). Lower numbers of ductules in PT area compared with D-septum is associated with the presence of micro-VI whereas a higher number is associated with absence of micro-VI (Fig. 5).

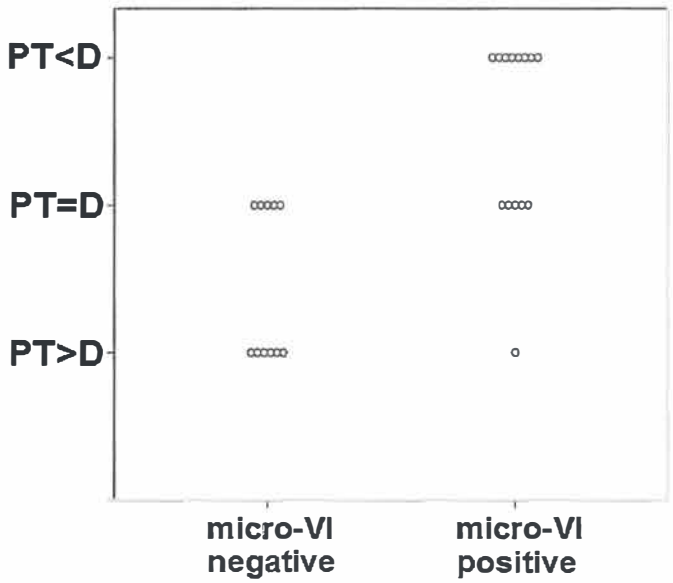

Figure 5. Distribution of the individual cases of the second cohort (n=25) in the 3 categories of the comparison between the PT and D-septa regarding the number of EpCAM positive ductules.
Micro-VI: microvascular invasion; PT: peritumoral septum; D: septum distant from the tumor.

Discussion

As a follow up of our previous study in which we found that the distinction between micro-VI positive and negative HCC was not only determined by tumor cell characteristics but also by differences in the tumor stroma, we performed the present study in which we focused on the PT stroma and D-septa. These stromal areas were isolated by LMD for gene expression analysis.

We found that PT septa of micro-VI positive and negative HCC did not show differences in gene expression of all tested angiogenic and EMT related factors as well as progenitor cell characteristics. A similar pattern was found for the D-septa. However, comparison of PT with D septa within the groups revealed a significant difference within the micro-VI positive group, but not in the micro-VI negative group, with regard to EpCAM and E-cadherin gene-expression. Both factors were significantly lower in the PT septa as compared with the expression in the D septa. As the protein expression of both factors could only be present in the ductular cells due to the absence of any other epithelial structures within the septa we concluded that differences in the number of ductules between the PT and D septa may underlie the pattern of the EpCAM and E-cadherin gene expression. EpCAM positive ductules in paraffin sections of PT and D septa were comparatively evaluated and a trend was observed toward an association between lower number of ductules in the PT septum and micro-VI positive HCC. To further validate the observed trend in the first cohort a similar comparative evaluation of EpCAM positive ductules were performed in a second and larger cohort of 25 HCC samples.

In this validation cohort it was found that lower number of ductules in PT septum relative to the corresponding D septum is significantly associated with the presence of micro-VI. Conversely, higher number of ductules in the PT septum compared to the D septum is associated with absence of micro-VI.

Our results are in line with the findings by Lennerz et al who investigated the ductules in HCC from the perspective of hepatocarcinogenesis [11]. They documented that loss of perinodular K19 positive ductules parallels the stepwise malignant transformation of cirrhotic nodules to high grade dysplasia and HCC and showed that the ductular loss is neither due to necrosis nor apoptosis. They also found increased numbers of cells that express the EMT markers Snail and S100A4 and suggested that this phenomenon contributes to hepatocarcinogenesis in a paracrine signaling manner. For instance, Snail expression is known to repress E-cadherin expression which will decrease intercellular adhesion and increase cellular detachment.

The mechanism proposed by Lennerz et al may also represent the underlying mechanism of the loss of ductules in our HCC samples although the

precise relationship between the possible ductular phenotypic switch with the presence of microvascular invasion remains to be elucidated.

The results of the present study and those of Lennerz et al showed the significance of the peritumoral area in hepatocarcinogenesis and invasive growth and corroborate the significance of the tumor microenvironment in cancer progression as has been outlined in recent reviews [9, 12].

From a diagnostic practical perspective our translational finding from gene expression levels into altered patterns of histological structures may lead to improvements of predicting micro-VI in HCC before surgery. Biopsies of HCC present in a cirrhotic liver that contain both PT and D septa will allow a similar comparative evaluation as described in this study. Together with other predictive parameters this evaluation may increase the reliability of predicting micro-VI as none of the reported predictive parameters contains reliably high predictive values that may justify therapeutic implications on a single parameter [5]. Robust validation studies are naturally necessary before application in routine practice.

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Chapter 5

Antiproliferative effects of sorafenib and pegylated IFN α-2b on human liver cancer cells *in vitro* and *in vivo*

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Abstract

Novel therapeutic strategies are needed to treat for patients with advanced hepatocellular carcinoma (HCC). Combination therapy of sorafenib and type I interferon (IFN) has substantial activity in patients with metastatic renal cell carcinoma. We investigated the antiproliferative effects of sorafenib in combination with pegylated interferon-α2b (PEG-IFN-α2b) on human hepatocellular carcinoma (HCC) cells *in vitro* and *in vivo*. A poorly differentiated HCC cell line derived from a patient with hepatitis C virus infection, HAK-1B, and a moderately differentiated HCC cell line, KIM-1, were used in this study. We demonstrated the synergistic antiproliferative effect of combination therapy on HAK-1B cells *in vitro*. In the *in vivo* study, the significant reduction of tumor volume and weight were observed in the combination group in both HAK-1B and KIM1 tumors, although synergistic effects were not clearly seen. CD34-positive-microvessel density was significantly lower and cleaved caspase-3-positive apoptotic cell number higher, in the sorafenib group and the combination group compared to control or PEG-IFN-α2b group in both HAK-1B and KIM-1 tumors. Ki67 labeling index was significantly lower in the combination group than in the control group in KIM-1 tumors. In conclusion, our results suggest that the combination therapy may be more effective for the treatment of HCC cases with variable sensitivity to anti-tumor effects of single therapy with either sorafenib or PEG-IFN-α2b.

Introduction

Primary liver cancer, of which hepatocellular carcinoma (HCC) represents the major subtype accounting to between 85% and 90%, is the sixth most common tumor globally, and the third most common cause of cancer-related death (1). Systemic treatment options for advanced HCC are limited and most deaths occurr within 1 year of diagnosis (2-4).

Sorafenib is an oral multikinase inhibitor that was approved by the U.S. Food and Drug Administration in December 2005 for the treatment of advanced renal cell carcinoma (RCC) and in November 2007 for the treatment of HCC. It has been shown to inhibit the activity of Raf kinase and several receptor tyrosine kinases, including vascular endothelial growth factor receptors (VEGFR)-1, 2, and 3, platelet-derived growth factor receptor (PDGFR)- α and β, FLT3, Ret and c-Kit. The intracellular signaling pathway Raf/MEK/ERK and the extracellular receptors VEGFR and PDGFR have been implicated in the molecular pathogenesis of HCC (5).

The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial revealed efficacy of sorafenib in the treatment of HCC, i.e., both median survival and time to progression showed 3-month improvements by sorafenib therapy (6). Cheng et al (7) also reported the efficacy of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma in 2009. Combination therapy with sorafenib has a potential to improve the outcome of sorafenib monotherapy. Phase II trial of combination therapy of sorafenib and IFN-α has substantial activity in patients with metastatic RCC (8,9). The combination therapy of IFN-α and 5-fluorouracil is partly or completely effective in about 50% of the patients with advanced HCC (10). Type I interferon (IFN) has various effects, including anti-viral effects, anti-proliferative effects and anti-angiogenic effects (11), and our laboratory previously reported the antiproliferative effect of IFN-α on human liver cancer cells *in vitro* and *in vivo* (12-14). In addition, type I IFN has suppressive effects on the occurrence of HCC, and the recurrence of HCC after curative treatment in patients with chronic hepatitis C virus infection (15-20). On the basis of above-described background, our current study examined the growth inhibitory effects of combination treatment of sorafenib and Pegylated IFN α-2b (PEG-IFN-α2b) on human HCC cell lines *in vitro* and *in vivo*.

Materials and methods

Cell line and cell cultures

This study used two HCC cell lines (KIM-1 (21) and HAK-1B (22)), which were originally established and characterized in our laboratory and previously confirmed to retain morphological and functional characteristics of the original tumor. Both of these two cell lines were established from surgically resected HCC nodules. KIM-1 is a moderately differentiated HCC cell line, and HAK-1B is a poorly differentiated HCC cell line which was derived from a patient with hepatitis C virus (HCV) infection. The cells were grown in Dulbecco's Modified Eagle Medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin (GIBCO BRL/Life Technologies Inc., Gaithersburg, MD) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37°C.

Sorafenib and pegylated IFNα-2b

Sorafenib, kindly provided by Bayer Pharmaceutical Corporation (West Haven, CT), was dissolved in dimethyl sulfoxide (DMSO) to create a 10 mM stock solution and stored at -20°C for *in vitro* study. For *in vivo* study, we prepared the solution at time of use. PEG-IFN-α2b (PEG Intron®) was kindly provided by MSD K.K. (Tokyo, Japan). The specific activity of PEG-IFN-α2b was 6.4×10^7 IU/mg protein.

Effect of sorafenib alone or combination treatment of sorafenib and PEG-IFN-α2b on the proliferation of HCC and CHC cell lines *in vitro*

The effects of sorafenib and/or PEG-IFN-α2b on the growth of the cultured cells were examined with colorimetry using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kits (Chemicon International Inc.) as described elsewhere (12-14). Briefly, the cells ($1.5–5.5 \times 10^3$ cells per well) were seeded on ninety-six-well plates (Nunc Inc., Roskilde, Denmark), cultured for twenty-four hours, and the culture medium was changed to a new one containing 0.2% DMSO (control) or Sorafenib (0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 μM), or both sorafenib (0, 1.25, 2.5 or 5 μM) and PEG-IFN-α2b (0, 2,000, 4,000, 8,000 IU/ml) (constant-ratio combination). After culturing for 72 hours, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optical density within linear range, all experiments were performed while the cells were in the logarithmic growth phase.

Combination analysis was performed by using the method as described by Chou and Talalay (23), and the CalcuSyn software program (Biosoft, Cambridge, UK) for automated analysis. This program calculates the combination index (CI). A CI of 0.9 - 1.1 indicates a nearly additive effect, a CI of less than 0.9 a synergistic effect, a CI of more than 1.1 an antagonistic effect.

Morphological observation

For morphological observation under a light microscope, cultured HAK-1B cells were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without 1.25 μM of sorafenib for seventy-two hours, fixed for ten minutes in Carnoy's solution, and stained with hematoxylin-eosin (HE).

Quantitative analysis of apoptotic cells induced by sorafenib and/or PEG-IFN-α2b

HAK-1B and KIM-1 were cultured with the culture medium containing 0.02% DMSO or 2 μM of sorafenib for seventy-two hours. For a study of combination therapy, HAK-1B cells were cultured with sorafenib (1.25 μM) or PEG-IFN-α2b (2,000 IU/ml), or both sorafenib (1.25 μM) and PEG-IFN-α2b (2,000 IU/ml) for seventy-two hours. After incubation, the cells were stained with the Annexin V-EGFP (enhanced green fluorescent protein) using Apoptosis Detection Kits (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the rate of Annexin V-EGFP-positive apoptotic cells was determined.

Effects of sorafenib and/or PEG-IFN-α2b on HCC cell proliferation in nude mice

This experiment was approved by the institutional committee for animal experiments and conducted according to the 'Guide for the Care and Use of Laboratory Animals' published and revised by the National Institute of Health in 1985.

Cultured HAK-1B or KIM-1 cells (1.0×10^6 cells/mouse) were transplanted subcutaneously (sc) to four-week-old female BALB/c athymic nude mice (Clea Japan Inc., Osaka, Japan). On the seventh day when tumor size became five to ten mm in diameter (Day 0), the mice were divided into four groups (n=8 each) in a manner to equalize the mean tumor diameter of every group. Each group was assigned to one of the four treatments: (A) control; (B) PEG-IFN-α2b alone; (C) sorafenib alone; (D) sorafenib + PEG-IFN-α2b (combination).

Sorafenib was diluted with 12.5% Cremophor EL/ 12.5% ethanol/ 75% water for oral dosing in mice. Sorafenib (200 μg/day) was administered by tube feeding once a day for fourteen days. PEG-IFN-α2b (1,920 IU) was subcutaneously injected twice a week for fourteen days (Day 1, Day 4, Day 8, and Day 11). In the control and the sorafenib alone groups, 0.1 ml of medium as the replacement of PEG-IFN-α2b was injected subcutaneously twice a week. In the control and the PEG-IFN-α2b alone groups, 0.2 ml of Cremophor EL/Ethanol/Water (12.5/12.5/75) as the replacement of sorafenib was administered by tube feeding once a day. The dose of sorafenib (200 μg) in the ratio to the average bodyweight of a mouse (20 g) was 10 mg/kg and this is almost comparable to a clinical dose (800 mg total daily dose). The clinical dose of PEG-IFN-α2b in chronic hepatitis C is 96,000 IU/kg per week. Because of species difference and different target which is not virus, but tumor, we used twice the dose per week in nude mice.

Tumor size was measured in two directions using calipers, and tumor volume (mm^3) was estimated by using the equation: length x $(width)^2$ x 0.5. This measurement was performed every two days. Mouse body weight was measured on Day 0, Day 7, and Day 14. Mouse was sacrificed and the tumor was resected on the next day after the completion of this fourteen day treatment (Day 15). The resected tumor was fixed in formalin after the weight measurement, prepared into paraffin sections, and underwent HE staining and immunohistochemistry.

Immunohistochemistry

Paraffin-embedded tissue samples were cut into four-micro meter sections. Anti-mouse CD34 (Rat monoclonal, MEC14.7, Abcam, Cambridge, UK) (1:50 dilution) and Ki67 (Rabbit monoclonal, SP6, Abcam, Cambridge, UK) (1:100 dilution) staining were performed by standard avidin-biotin-peroxidase complex method and 3,3'-diaminobenzidine (DAB) solution was used for color development. Cleaved caspase-3 (rabbit polyclonal antibody, Cell Signaling Technologies, Beverly, MA, USA) (1:250 dilution) staining was performed on the Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) to detect the apoptotic cells. This automated system uses the streptavidin-biotin complex method with DAB as a chromogen (Ventana iVIEW DAB Detection KIT).

Microvessel density (MVD) was evaluated within the tumor according to a modified method introduced by Tanigawa et al (24). Briefly the slides stained with CD34 were screened at low power field (x40 or x100) and the two or three most vascular areas were selected. Microvessel counts of these areas were performed at high power field (x200, 0.74 mm^2). All positive stained cells were counted as microvessels and every 40 μm length of vessel lumen was calculated as one point. The average microvessel counts of selected areas were regarded as MVD, which

was expressed as the absolute number of microvessels per 0.74 mm^2. Immunohistochemically, cleaved caspase-3 was expressed perinuclearly and Ki67 was on nuclear. The rate of apoptotic cells and Ki67 labeling index were evaluated by calculating the rate of cleaved caspase-3-positive cells and Ki67-positive cells, respectively.

Statistical analysis

Comparisons of estimated tumor volume and colorimetric cell growth were performed using two-factor factorial ANOVA and Student's t-test, respectively. The other data comparisons were performed using the Mann-Whitney U-test.

RESULTS

Effect of sorafenib alone or combination treatment of sorafenib and PEG-IFN-α2b on the proliferation of HAK-1B or KIM-1 HCC cells *in vitro*

Seventy-two hours after the addition of sorafenib, the relative viable cell number was suppressed in both HAK-1B and KIM-1 cell lines in a dose-dependant manner (Fig. 1). The 50% inhibitory concentration (IC50) was 2.1 μM for HAK-1B and 2.5 μM for KIM-1.

Seventy-two hours after the addition of PEG-IFN-α2b and sorafenib, the relative viable cell number was suppressed to various degrees. The results are exhibited in Fig. 2. In HAK-1B cell line (Fig. 2A), significant difference in the relative viable cell number was observed between combination group and sorafenib or PEG-IFN-α2b alone groups, additionally, CI in all combination of PEG-IFN-α2b and sorafenib was less than 0.9. The CI was 0.879 in the combination of 2,000 IU/ml of PEG-IFN-α2b and 1.25 μM of sorafenib, 0.667 in 4,000 IU/ml of PEG-IFN-α2b and 2.5 μM of sorafenib, and 0.842 in 8,000 IU/ml of PEG-IFN-α2b and 5.0 μM of sorafenib. According to the definition of the CI, these results indicate that a combination of PEG-IFN-α2b and sorafenib may produce a synergistic growth inhibitory effect in HAK-1B cell line. In KIM-1 cell line (Fig. 2B), there was also a significant difference in the relative viable cell numbers between combination group and monotherapy groups. The CI was 0.912 in the combination of 2,000 IU/ml of PEG-IFN-α2b and 1.25 μM of sorafenib, 0.992 in 4,000 IU/ml of PEG-IFN-α2b and 2.5 μM of sorafenib, and 0.823 in 8,000 IU/ml of PEG-IFN-α2b and 5.0 μM of sorafenib. These results indicate that combination therapy may produce an additive or synergistic growth inhibitory effect in KIM-1 cell line.

Morphologically, HAK-1B cells showed characteristic features of apoptosis, such as cytoplasmic shrinkage and nuclear chromatin condensation at seventy-two hours after adding 1.25 μM of sorafenib (Fig. 3).

The rate of Annexin V-EGFP positive apoptotic cells was increased by adding 2 μM of sorafenib in HAK-1B cells (5.8% of the control and 37.8% of the sorafenib). In KIM-1 cells, however, the increase was relatively small (7.9% of the control and 9.5% of the sorafenib) (Fig. 4A). In another setting, the combination group with PEG-IFN-α2b showed higher rate of apoptosis than control or monotherapy groups in HAK-1B (4.8% of control, 37.4% of the PEG-IFN-α2b, 14.3% of the sorafenib, 42.8% of the combination) (Fig. 4B).

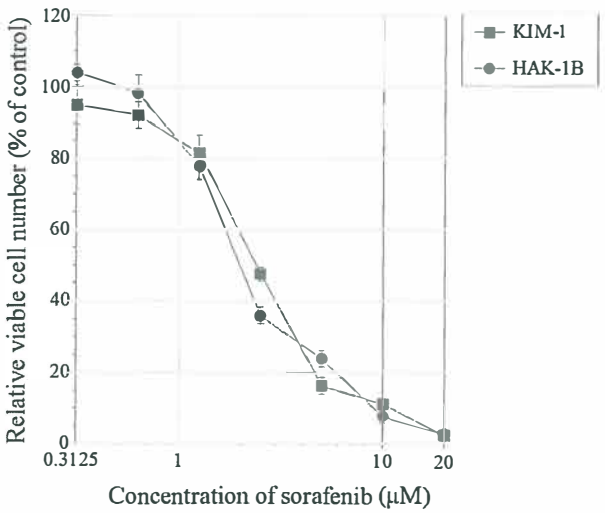

Figure 1. Seventy-two hours after adding 0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 μM of sorafenib. Cell proliferation was suppressed in a dose-dependant manner in both KIM-1 and HAK-1B cell lines. The suppression was significant (P<0.001-0.05) in the range of 0.625-20 μM of sorafenib in KIM-1, 1.25-20 μM in HAK-1B. 50% growth inhibitory dose was 2.5 μM in KIM-1 and 2.1 μM in HAK-1B. The values represent mean ± SD.

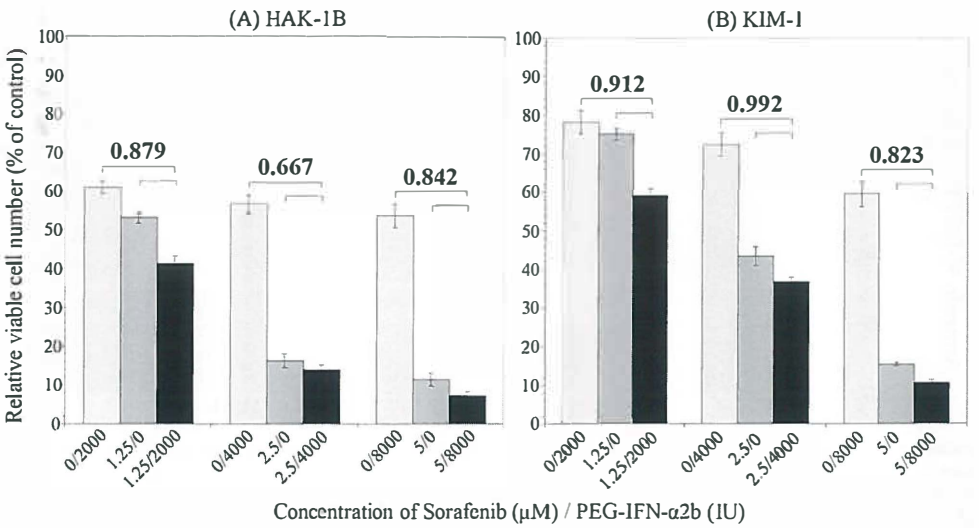

Figure 2. Effect of PEG-IFN-α2b and/or sorafenib on the proliferation of human HCC cell lines HAK-1B (A) and KIM-1 (B) in culture for 72 hours. Light gray bars are PEG-IFN-α2b alone group, dark gray bars sorafenib alone group, and black bars PEG-IFN-α2b + sorafenib group. All combination groups showed significant difference compared with monotherapy groups. The numbers above bars are CI. A CI of 0.9 – 1.1 indicates a nearly additive effect, a CI of less than 0.9 a synergistic effect, a CI of more than 1.1 an antagonistic effect. Representative data of two independent experiments were shown. The values represent mean ± SD.

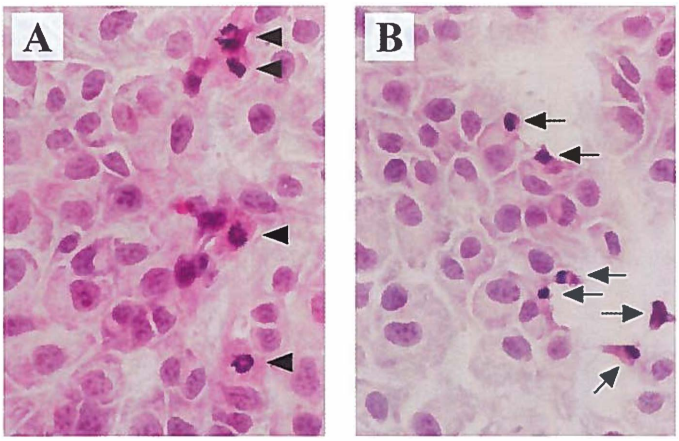

Figure 3. Photomicrograph of HAK-1B cells cultured for seventy-two hours on Lab-Tek Chamber slide. (A) Without sorafenib in culture medium. Some mitotic figures were noted (arrow heads). (B) With 1.25 μM of sorafenib in culture medium. There were some apoptotic cells characterized by cytoplasmic shrinkage and nuclear chromatin condensation (arrows).

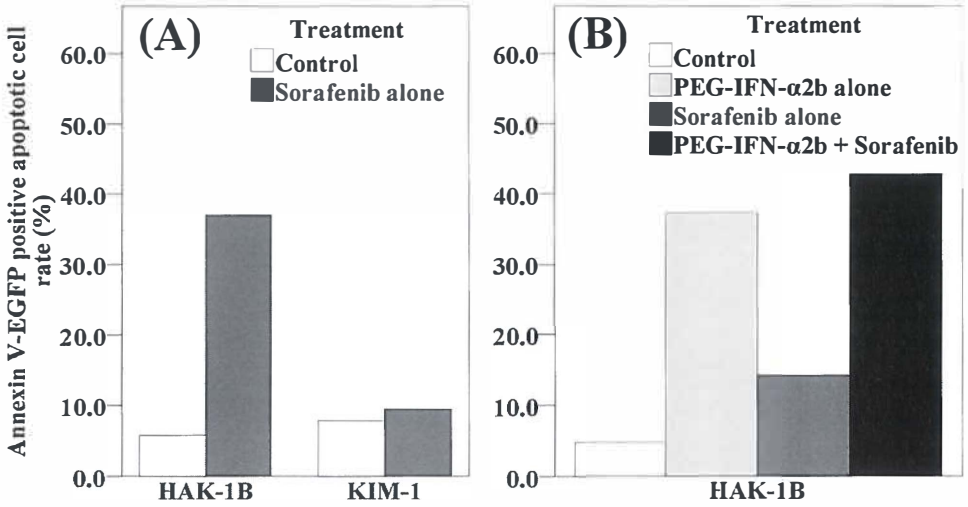

Figure 4. Quantitative analysis of Annexin V-EGFP positive apoptotic cells. (A) Apoptosis of HAK-1B or KIM-1 cells induced by 2 μM of sorafenib. (B) Apoptosis of HAK-1B cells induced by 2,000 IU/ml of PEG-IFN-α2b and/or 1.25 M of sorafenib. Representative data of three independent experiments were shown.

Effects of sorafenib and/or PEG-IFN-α2b on HAK-1B or KIM-1 cell proliferation in nude mice

Chronological changes in estimated tumor volume after subcutaneous injection of cultured HAK-1B cells or KIM-1 cells to nude mice are summarized in Fig. 5. The actual tumor weights at the time of sacrifice are exhibited in Table 1. In the experiment of HAK-1B tumors, the tumor volume of mice receiving PEG-IFN-α2b, sorafenib, and sorafenib+PEG-IFN-α2b was 34%, 73%, and 36%, respectively, of the control volume and the tumor weight was 23%, 71%, and 34%, respectively, of the control weight. Statistically, there were significant differences both in tumor volume and weight between the control group and the PEG-IFN-α2b alone group ($P<0.0001$ versus control in tumor volume, $P<0.0001$ versus control in tumor weight) or the combination group ($P<0.0001$ versus control in tumor volume, $P<0.001$ versus control in tumor weight) and between the sorafenib alone group and the PEG-IFN-α2b alone group ($P<0.005$ versus sorafenib alone in tumor volume, $P<0.05$ versus sorafenib alone in tumor weight). Although there was a significant difference between the sorafenib alone group and the combination group in tumor volume ($P<0.001$), there was not in actual tumor weight ($P=0.099$). In the experiment of KIM-1 tumors, the tumor volume of mice receiving PEG-IFN-α2b, sorafenib, and sorafenib+PEG-IFN-α2b was 69%, 45%, and 46%, respectively, of the control volume and the tumor weight was 75%, 41%, and 37%, respectively, of the control weight. Statistically, there were significant differences in both tumor volume and weight between the control and the sorafenib alone group ($P<0.0001$ versus control in tumor volume, $P<0.05$ versus control in tumor weight) or the combination group ($P<0.001$ versus control in tumor volume, $P<0.05$ versus control in tumor weight).

The results of immunohistochemical examination are summarized in Table 2. The significant decrease of MVD and increase of apoptotic cells were observed in the sorafenib group ($P<0.0005$ and 0.05 respectively versus control in HAK-1B, $P<0.05$ and 0.05 respectively versus control in KIM-1) and the combination group ($P<0.05$ and 0.05 respectively versus control in HAK-1B, $P<0.05$ and 0.05 respectively versus control in KIM-1) compared to the control group in both HAK-1B and KIM-1 tumors, although there was no significant difference between the combination group and monotherapy groups. Ki67 labeling index was significantly lower in the combination group ($P<0.005$ versus control, $P<0.05$ versus PEG-IFN-α2b group) than in the control group or the PEG-IFN-α2b group only in KIM-1.

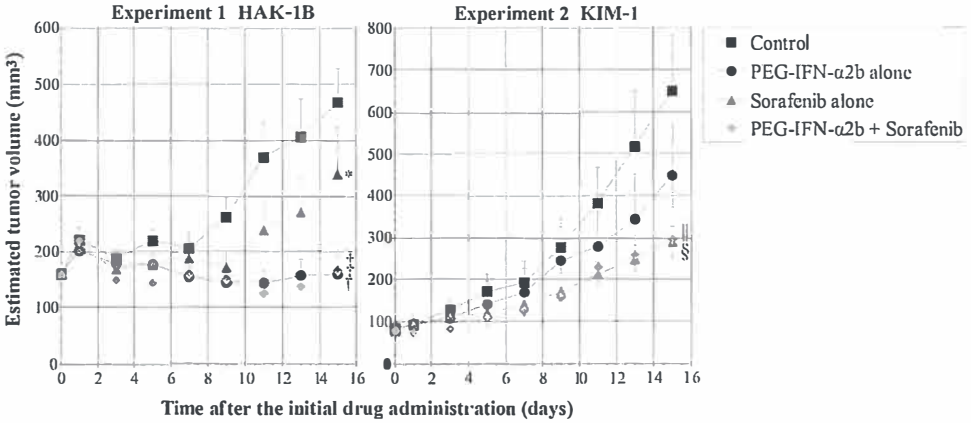

Figure 5. Chronological changes on the estimated volume of HAK-1B (Experiment 1) or KIM-1 (Experiment 2) tumor that was developed subcutaneously on nude mice. The PEG-IFN-α2b alone group (●) received subcutaneous injection of 1,920 IU twice a week for 14 days. The sorafenib alone group (▲) received 10 mg/kg/mouse/day orally every day for 14 days. The PEG-IFN-α2b + sorafenib group (♦) received 1,920 IU of PEG-IFN-α2b twice a week and 10 mg/kg of sorafenib every day for 14 days. The control group (■) received subcutaneous injection of 0.1 ml of medium twice a week and 0.2 ml of Cremophor EL/Ethanol/Water (12.5/12.5/75). The values represent mean ± SE. *P<0.05 versus control. †P<0.0001 versus control, P<0.005 versus sorafenib alone. ‡P<0.0001 versus control, P<0.001 versus sorafenib alone. §P<0.001 versus control. ||P<0.0001 versus control.

Table 1. The weight of subcutaneous tumor of HAK-1B cells or KIM-1 cells in nude mice at sacrifice.

	Tumor weight (g)	
Treatment group	HAK-1B	KIM-1
Control	0.333 ± 0.03	0.504 ± 0.17
PEG-IFN-α2b alone	0.078 ± 0.02 *	0.379 ± 0.18
Sorafenib alone	0.236 ± 0.06	0.206 ± 0.04 ***
PEG-IFN-α2b + sorafenib	0.113 ± 0.04 **	0.185 ± 0.12 ***

Tumor weight represents mean ± SE (g). * P<0.0001 versus control, P<0.05 versus sorafenib alone. ** P<0.001 versus control. *** P<0.05 versus control.

Table 2. MVD and the ratio of apoptotic cells and Ki67 positive cells in human HCC tumors subcutaneously transplanted in nude mice.

Cell line	Treatment group	MVD	Apoptotic cells	Ki67 positive cells
HAK-1B	Control	100.8 ± 7.7	3.8 ± 0.4	36.8 ± 2.0
	Peg-IFN-α2b alone	114.9 ± 16.7	4.4 ± 0.4	37.5 ± 4.6
	Sorafenib alone	53.8 ± 4.3 *	6.7 ± 1.3 **	38.3 ± 2.0
	Peg-IFN-α2b+soragfenib	69.4 ± 10.1 **	5.6 ± 1.3 **	35.3 ± 2.2
KIM-1	Control	125.9 ± 16.2	4.6 ± 0.4	6.7 ± 0.2
	Peg-IFN-α2b alone	97.4 ± 10.4	5.1 ± 0.4	7.5 ± 0.8
	Sorafenib alone	85.1 ± 6.6 **	6.5 ± 0.7 **	5.7 ± 0.4
	Peg-IFN-α2b+soragfenib	79.0 ± 7.2 **	6.3 ± 0.6 **	4.6 ± 0.5 ***

Scores represent mean ± SE. * P<0.0005 versus control, P<0.01 versus Peg-IFN-α2b alone. ** P<0.05 versus control. *** P<0.005 versus control, P<0.05 versus Peg-IFN-α2b alone.

DISCUSSION

In this study, we showed the synergistic effect of sorafenib and PEG-IFN-α2b on HAK-1B cells *in vitro*. We previously reported that PEG-IFN-α2b induced apoptosis on both HAK-1B and KIM-1 cells *in vitro* (14). We found that sorafenib also induced apoptosis on HAK-1B *in vitro*. On the other hand, the increase of apoptotic cells was not clearly observed on KIM-1 cells in spite of the fact that the proliferation of KIM-1 cells was inhibited by sorafenib in MTT assay. A possible explanation is that cell proliferation might be inhibited by other anti-proliferative mechanisms. The blockade of Raf signaling which is main effect of sorafenib can lead to the repression of transforming growth factor α-epidermal growth factor receptor autocrine loops of tumor cells (5). Such a mechanism could have inhibited the growth of KIM-1 cells. In addition, a limitation of *in vitro* study is that we are not able to assess the indirect anti-angiogenic effect against endothelial cells.

In the *in vivo* study, there was a significant reduction of tumor volume and weight in the combination group on both HAK-1B and KIM-1 tumors compared with the control group. However there was no significant difference between the combination and the monotherapy groups, and it seemed that HAK-1B tumors were sensitive to PEG-IFN-α2b and KIM-1 tumors to sorafenib. Only in KIM-1 tumors that might be sensitive to sorafenib, Ki67 labeling index was lower in the combination group than in the control group. Recently Wang et al (25) reported that combination therapy of sorafenib with recombinant human INF-α2a was effective *in vitro* and *in vivo* on two HCC cell lines, Huh-7 and Sk-Hep-1. In their study, the significant differences between combination and monotherapy groups were clearly observed. This partial difference might be due to the different experimental settings, such as different cell lines and different dose of drugs. One of the biggest differences, we surmise, is the site of IFN administration. They injected IFN directly into subcutaneous tumors, whereas we did subcutaneously but not into the tumors.

Since sorafenib is a multikinase inhibitor, it is considered that sorafenib has both direct anti-proliferative effect due to the blockade of Raf kinase on tumor cells themselves and indirect effect due to the blockade of receptor tyrosine kinases, such as VEGFR-2, on endothelial cells followed by the inhibithion of angiogenesis (5). Therefore we also evaluated MVD of xenografts and confirmed the significant decrease of MVD in the sorafenib alone and the combination group in both HAK-1B and KIM-1 tumors. It has been repeatedly shown that IFN suppress the growth of the various types of human tumor that was transplanted to mice through the anti-angiogenic effect. For instance, Tedjarati et al (26) reported that the subcutaneous injection of 7,000 IU per week of PEG-IFN-α2b into nude mice bearing human ovarian cancer cells induced the significant decrease of CD31 positive endothelial cells and Huang et al (27) also showed the similar results with

the subcutaneous injection of 70,000 IU per week of PEG-IFN-α2b on human prostate cancer cells. PEG-IFN-α2b administered at higher or lower doses was less effective. In our current study, however, there was no significant decrease of MVD in the PEG-IFN-α2b group compared with the control group. Moreover, in our previous report, the decrease of artery-like blood vessels was not observed in the same HAK-1B tumors by the administration of PEG-IFN-α2b at either higher or lower doses (14).

Another notable finding regarding to MVD in this study is the discrepancy between MVD and tumor weight or size. Interestingly, the reduction of tumor weight and size was not so much in sorafenib monotherapy group in HAK-1B tumors despite the most prominent decrease of MVD was observed in this group. On the other hand, there was a significant reduction of tumor weight and size in PEG-IFN-α2b alone group in HAK-1B, although this group did not show any significant decrease of MVD. This result supports our previous findings in which we showed there was no relationship between tumor shrinkage and the number of artery-like blood vessels in HAK-1B tumors after the administration of the various concentration of PEG-IFN-α2b (14). Hlatky et al (28) mentioned in their review article that the efficacy of anti-angiogenic agents cannot be simply visualized by alterations in microvessel density during treatment because of the difference of the tightness of the coupling between vessel dropout and tumor-cell dropout after the treatment. In addition, Yao et al (29) recently reported that the expression of VEGFR-1 in tumor cells which is normally expressed specifically in endothelial cells were strongly associated with anti-PIGF antibodies efficacy, but not with antiangiogenesis. More studies are needed to investigate more appropiate ways to assess the efficacy of anti-angiogenic drugs *in vivo* and molecular mechanisms of their action of "anti-angiogenic" drugs.

In conclusion, we demonstrated the synergistic antiproliferative effect of combination therapy on HAK-1B cells *in vitro*. Although, *in vivo* study, synergistic effects of the combination therapy were not clearly observed, the combination therapy induced nearly maximum anti-tumor effects, independent of the HCC cells' sensitivity to anti-tumor effects of single therapy with either PEG-IFN-α2b or sorafenib. These findings suggest that PEG-IFN-α2b might be a promising candidate for use in combination therapy with sorafenib and warrant further investigation.

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Chapter 6

Pegylated interferon-α2a inhibits proliferation of human liver cancer cells *in vitro* and *in vivo*

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Abstract

Purpose: We investigated the effects of pegylated interferon-α2a (PEG-IFN-α2a) on the growth of human liver cancer cells.

Methods: The effect of PEG-IFN-α2a on the proliferation of 13 liver cancer cell lines was investigated *in vitro*. Cells were cultured with medium containing 0–4,194 ng/mL of PEG-IFN-α2a, and after 1, 2, 3, or 4 days of culture, morphologic observation and growth assay were performed. After hepatocellular carcinoma (HCC) cells (HAK-1B and KIM-1) were transplanted into nude mice, various doses of PEG-IFN-α2a were subcutaneously administered to the mice once a week for 2 weeks, and tumor volume, weight, and histology were examined.

Results: PEG-IFN-α2a inhibited the growth of 8 and 11 cell lines in a time- and dose-dependent manner, respectively, although the 50% growth inhibitory concentrations of 7 measurable cell lines on Day 4 were relatively high and ranged from 253 ng/mL to 4,431 ng/mL. Various levels of apoptosis induction were confirmed in 8 cell lines. PEG-IFN-α2a induced a dose-dependent decrease in tumor volume and weight, and a significant increase of apoptotic cells in the tumor. Subcutaneous administration of clinical dose for chronic hepatitis C (3 μg/kg, 0.06 μg/mouse) was effective and induced about 30-50% reduction in the tumor volume and weight as compared with the control.

Conclusions: Although *in vitro* anti-proliferative effects of PEG-IFN-α2a were relatively weak, PEG-IFN-α2a induced strong anti-tumor effects on HCC cells *in vivo*. The data suggest potential clinical application of PEG-IFN-α2a for the prevention and treatment of HCC.

Introduction

Interferons (IFNs) are types of cytokine that are produced by host cells, such as leukocytes, in response to inflammation. Since IFNs possess antiviral activity, antiproliferative activity and various immunoregulatory activities, IFN therapy is used to treat patients with chronic viral hepatitis or certain types of cancer including malignant melanoma, acquired immunodeficiency syndrome-related Kaposi's sarcoma and some hematopoietic malignancies [1, 2]. Lai et al also showed that recombinant IFNα is useful in prolonging survival among patients with inoperable hepatocellular carcinoma (HCC) [3]. In addition, some studies showed IFN therapy might prevent either occurrence or recurrence after initial curative therapy of HCC, such as liver resection and radiofrequency ablation, in patient with chronic viral hepatitis [4–7]. This cancer preventive effect of IFNs is regarded mainly as results of their antiviral effect and the consequent suppression of inflammation, and might be due to their direct antitumor effect against clinically undetectable HCC as well. The detailed mechanism of the antitumor effect of IFNs, however, remains obscure.

Pegylated interferon-α2a (PEG-IFN-α2a) and pegylated interferon-α2b (PEG-IFN-α2b), which are used to treat patients with chronic hepatitis C virus (HCV) or B virus (HBV) infection, are modified IFNs that have longer serum half-life in body than non-pegylated forms of IFNs, therefore they can be given to patients only once a week, whereas a standard IFN without pegylation used to be injected up to three to five times a week. This once-a-week injection of pegylated IFNs in combination with daily oral dosing of the nucleoside analogue ribavirin has substantially improved the rate of sustained virological response in patients with chronic HCV infection and got a position as the first line therapy [8, 9]. We previously reported that PEG-IFN-α2b which contains 12 kDa polyethylene glycol (PEG) has stronger antitumor effects *in vivo* than non-pegylated IFNs and this result might be indicating that continuous IFNs exposure to cancer cells in body is more effective than continual injection [10]. On the basis of above-described background, we examined the growth inhibitory effects of PEG-IFN-α2a which contains two chains of 20 kDa PEG and has the longest serum half-life among clinically available IFNs on liver cancer cell lines *in vitro* and *in vivo*.

Methods

Cell Lines and Cell Culture

This study used 11 HCC cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B, HAK-2, HAK-3, HAK-4, HAK-5, and HAK-6) and 2 human combined

hepatocellular and cholangiocarcinoma (CHC) cell lines (KMCH-1 and KMCH-2). These HCC and CHC cell lines were originally established in our laboratory, and each cell line retains the morphological and functional features of the original tumor as described elsewhere [11−20].

The cells were grown in Dulbecco's Modified Eagle Medium (Nissui Seiyaku, Co., Japan) supplemented with 2.5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Bioserum, Victoria, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO BRL/Life Technologies, Inc., Gaithersburg, MD) and 12 mmol/L sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37°C.

IFN and Reagents

PEG-IFN-α2a (PEGASYS®, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) with the specific activity of 1.4 X 10^7 IU/mg protein and non-pegylated IFN-α2a (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with that of 2.0 X 10^8 IU/mg protein were used in the study.

Anti-bromodeoxyuridine (BrdU) antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (FITC-GAM) were purchased from Becton Dickinson Immunocytometry Systems USA (San Jose, CA); control normal mouse IgG_1, from DAKO (Glostrup, Denmark); rat antibody against mouse endothelial cells (anti-CD34, clone MEC14.7), from Serotec Co., UK; and mouse monoclonal antibody against human α-smooth muscle actin (SMA) that cross-reacts with mouse α-SMA (clone 1A4).

Effects of PEG-IFN-α2a on the Proliferation of HCC and CHC Cell Lines *in vitro*

The effects of PEG-IFN-α2a on the growth of the cultured cells were examined with colorimetry using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kits (Chemicon, Temecula, CA) as described elsewhere [18, 21]. Briefly, the cells (1.5~8 X 10^3 cells per well) were seeded on 96-well plates (Nunc, Inc, Roskilde, Denmark), cultured for 24 hours, and the culture medium was changed to a new medium with or without PEG-IFN-α2a (0.016, 0.064, 0.256, 1.024, 4.096, 16.4, 65.5, 262, 1,048, or 4,194 ng/mL). After culturing for 24, 48, 72 or 96 hours, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optical density within linear range, all experiments were performed while the cells were in the logarithmic growth phase.

Quantitative analysis of apoptotic cells induced by PEG-IFN-α2a

HAK-1B or KIM-1 cells cultured with medium alone (control), non-pegylated IFN-α2a (10 ng/ml=2,000 IU/ml) or PEG-IFN-α2a (144 ng/ml=2,000 IU/ml) for 72 hours

were stained with the Annexin V-EGFP (enhanced green fluorescent protein) Apoptosis Detection Kits (Medical & Biological Laboratories Co., Ltd.) according to the manufacturer's instructions. After staining, the cells were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), and Annexin V-EGFP-positive apoptotic cell rate was determined.

Morphological Observation

For morphological observation under a light microscope, cultured cells were seeded on Lab-Tek tissue culture chamber slides (Nunc, Inc.), cultured with or without PEG-IFN-α2a (262, 1,048 or 4,194 ng/mL) for 72 hours, fixed for 10 min in Carnoy's solution, and stained with hematoxylin-eosine (HE).

Effects of PEG-IFN-α2a on HCC Cell Proliferation in Nude Mice

All animal experiments were approved by the institutional committee for animal experiments in Kurume University School of Medicine (Permit Number: 1334), and conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and the Regulations for Animal Experimentation of Kurume University School of Medicine. Mice were killed by cervical dislocation under diethyl ether anesthesia, and all efforts were made to minimize suffering. Cultured HAK-1B or KIM-1 (10^7 cells/mouse) was subcutaneously (s.c.) injected into the backs of 5-week-old female BALB/c athymic nude mice (Clea Japan, Inc., Osaka, Japan). Five to seven days later when the largest diameter of the tumor, which was measured by using caliper, reached approximately 5~10 mm (Day 0), tumor volume (mm^3) was culculated in the equation 'the largest diameter X (the smallest diameter)^2 X 0.5', and then the mice were divided into 5 groups (n=8 each). The mean tumor volume of each group was equalized. Tumor volume was measured on Day 0, 1, 2, 4, 6, 8, 10, 12, and 14. Mouse body weight was measured on Day 0, 8, and 14. After 2-week treatment, mice were killed on Day 15 and the actual tumor weight was also measured. In experiment #1, each mouse received a subcutaneous injection of 0.333 mL of phosphate-buffered saline (PBS) (Control), PBS containing 0.06, 0.6, 6 or 60 μg (840, 8,400, 84,000, or 840,000 IU, respectively) of PEG-IFN-α2a once a week for 2 consecutive weeks (Day 1 and Day 8). The clinical dose of PEG-IFN-α2a in chronic hepatitis C treatment is about 3 μg/kg and is equivalent to the lowest dose (0.06 μg/mouse=840 IU/mouse) in this experiment. After killing, resected tumors were used for morphological studies (e.g., HE staining and immunohistochemistry) and Enzyme-linked immunosorbent assay (ELISA) analysis. Every mouse received an intraperitoneal injection of 1 mg of BrdU 30 min before killing. In experiment #2, to examine the difference between non-pegylated and pegylated IFNs, each mouse received a subcutaneous injection of 0.333 mL of PBS (Control), PBS containing 0.06 or 0.6 μg of PEG-IFN-α2a (840

or 8,400 IU, respectively), or PBS containing 0.0042 or 0.042 μg of IFN-α2a (840 or 8,400 IU, respectively). In this experiment, tumor weights on Day 15 were compared among the groups.

Morphological Examination of the Subcutaneous Tumors of Nude Mice

The number of cells showing the characteristics of apoptosis (e.g., cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation) was counted in at least three 0.25 mm^2-areas within an HE-stained specimen, and the average number per area was obtained. The TUNEL technique (ApopTag® Peroxidase *In Situ* apoptosis Detection Kits, CHEMICON International, Inc, CA) was used to detect apoptotic cells, and the average number of TUNEL-positive cells per area was obtained, as described above. The specimens were also immunostained for incorporated BrdU using BrdU Staining Kits (Oncogene Research Products, Boston, MA), and the average number of positive cells per area was obtained as described above. In addition, double-immunostaining was performed with anti-mouse endothelial cell antibody, anti-human α-SMA antibody, Histofine simple stain mouse MAX-PO (Rat) kits (Nichirei, Tokyo, Japan), and HistoMouse™-plus kits to detect artery-like blood vessels as described in our previous report [21, 22]. The number of double-immunostaining-positive blood vessels in the tumor and in the borderline area between the tumor nodule and surrounding tissues was counted on each specimen. The size of the counted area was measured by tracing the outline displayed on a computer monitor using Mac SCOPE (MITANI CORP., Chiba, Japan). From the obtained number of vessels per unit area (mm^2), the group mean was obtained for group comparison.

Enzyme-linked immunosorbent assay (ELISA)

Portions of the resected xenograft tumors were homogenized in 500 μl of ice-cold Ca^{2+} and Mg^{2+}-free PBS containing 100 mg/ml phenymethylsulfonyl fluoride using a pellet pestle. The mixture was centrifuged for 10 min (12,000 g, 4°C), and the supernatant was stored at -20°C until use. After the determination of the amount of the tissue protein in the supernatant using a BCA protein assay reagent (Pierce, Rockford, IL), the amount of basic fibroblast growth factor (bFGF) and IL-8 was measured by using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Statistics

Comparisons of estimated tumor volume and colorimetric cell growth were performed using two-factor factorial ANOVA and Student's *t*-test, respectively. The other data comparisons were performed using the Mann-Whitney U test.

Results

Effects of PEG-IFN-α2a on Liver Cancer Cell Proliferation *in vitro*

Twenty-four hours after the addition of 4,194 ng/mL of PEG-IFN-α2a, mild increase in the relative viable cell number occurred in 9 cell lines (all cell lines except KYN-2, HAK-1A, HAK-6, and KMCH-1). However, after 72 hours or later, a 10% or more decrease in the cell number occurred in all cell lines (Fig. 1A). In HAK-2, HAK-3, and HAK-4, HAK-6, and KMCH-2, proliferation was suppressed up to 72 hours and the cell number reached a plateau or slightly increased thereafter. In the other 8 cell lines, proliferation was suppressed to varying degrees up to 96 hours.

The relative viable cell number was suppressed in 11 cell lines (all cell lines except HAK-1A and KMCH-2) in a dose-dependent manner after the 96 hours-incubation with PEG-IFN-α2a (Fig. 1B). In 7 cell lines (HAK-1B, KMCH-1, KIM-1, KYN-1, HAK-6, KYN-3, and KYN-2), the number was suppressed to 50% or less with 4,194 ng/mL of PEG-IFN-α2a, and the 50% inhibitory concentration (IC50) was 253 ng/mL for HAK-1B, 670 ng/mL for KMCH-1, 1,105 ng/mL for KIM-1, 1,128 ng/mL for KYN-1, 1,302 ng/mL for HAK-6, 1,524 ng/mL for KYN-3, and 4,431 ng/mL for KYN-2. No relationship was detected between the histological differentiation level of the original tumor and sensitivity to the anti-proliferative effect of PEG-IFN-α2a.

Seventy-two hours after adding 4,194 ng/mL of PEG-IFN-α2a, 8 cell lines (all cell lines except KYN-3, HAK-1A, HAK-2, HAK-3, and KMCH-2) showed characteristics of apoptosis, e.g., cytoplasmic shrinkage, chromatin condensation, and nuclear fragmentation, in various degrees and in a dose-dependent manner (Fig. 2). The appearance of apoptosis was further confirmed in HAK-1B and KIM-1 cells cultured with 10 ng/ml (=2,000 IU/ml) of IFN-α2a or 144 ng/ml (=2,000 IU/ml) of PEG-IFN-α2a by apoptosis detection assay (Table 1). Non-pegylated IFN-α2a induced much more apoptosis than PEG-IFN-α2a.

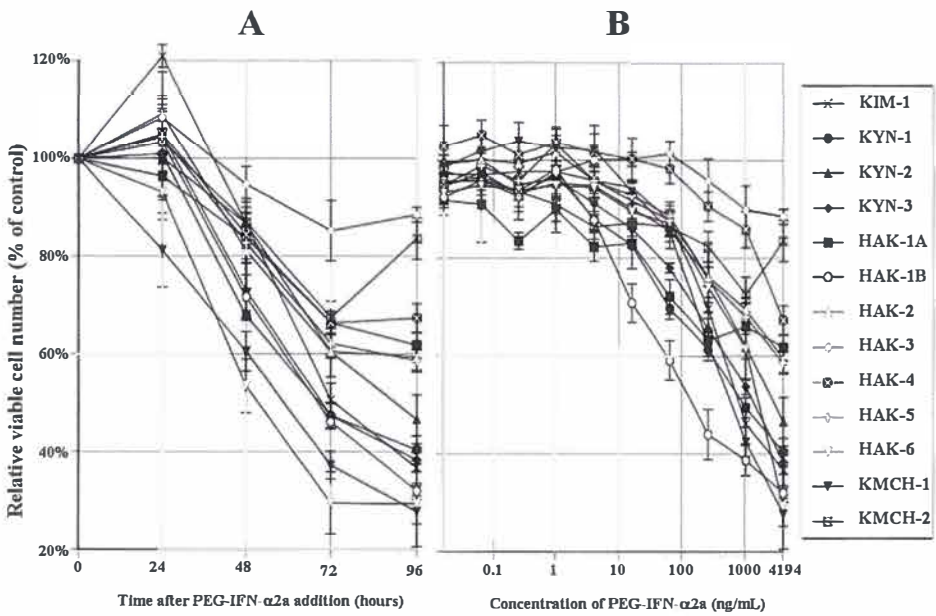

Figure 1. Anti-proliferative effect of PEG-IFN-α2a. (A) Chronological changes in relative viable cell number (% of the control) after adding 4,194 ng/mL of PEG-IFN-α2a. Growth was suppressed with time in 8 cell lines. (B) 96 hours after adding 10 different concentrations of PEG-IFN-α2a. Cell proliferation was suppressed in a dose-dependent manner in 11 cell lines. The suppression was significant (P < 0.0001~0.05) in the ranges of 0.016~4,194 ng/mL of PEG-IFN-α2a in HAK-6, 0.256~4,194 ng/mL in KYN-3 and HAK-1A, 4.096~4,194 ng/mL in KIM-1, KYN-1, HAK-1B, HAK-2 and KMCH-2, 16.4~4,194 ng/mL in KYN-2, HAK-5 and KMCH-1, 262~4,194 ng/mL in HAK-4, and at 4,194 ng/mL in HAK-3 (Student t-test). Eight samples were used in each experiment (n = 8). The experiment was repeated at least 3 times for each cell line. The figures represent average ± SE of the experiments.

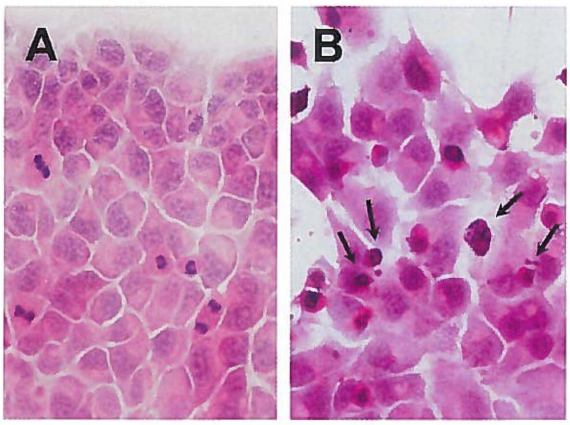

Figure 2. Photomicrograph of HAK-1B cells cultured for 72 hours on a Lab-Tek Chamber slide. (A) Without PEG-IFN-α2a in culture medium. (B) With 4,194 ng/mL of PEG-IFN-α2a in culture medium. Apoptotic cells (short arrows) characterized by cytoplasmic shrinkage, chromatic condensation and nuclear fragmentation were noted (HE staining, X 200).

Table 1. Quantitative analysis of apoptosis in HAK-1B or KIM-1

	Annexin V-EGFP apoptotic cells (%)		
Cell line[a]	Control	IFN-α2a	PEG-IFN-α2a
HAK-1B	4.1 ± 0.5[b]	18.5 ± 0.3	10.9 ± 0.5
KIM-1	9.4 ± 0.4	47.0 ± 0.2	29.8 ± 2.1

[a] Cells were cultured with medium alone (Control), IFN-α2a (10 ng/ml=2,000 IU/ml) or PEG-IFN-α2a (144 ng/ml=2,000 IU/ml). [b] Mean ±SE.

Effects of PEG-IFN-α2a on HCC Cell Proliferation in Nude Mice

Chronological changes in estimated tumor volume after subcutaneous injection of cultured HAK-1B or KIM-1cells to nude mice are summarized in Fig. 3. Dose-dependent suppression of tumor volume was observed in mice receiving PEG-IFN-α2a. In the experiment of HAK-1B tumors, a significant difference in the changes in tumor volume and tumor weight was observed between the Control mice and the mice that received 0.06, 0.6, 6 or 60 μg of PEG-IFN-α2a ($P < 0.0001$ by two-factor factorial ANOVA; and $P < 0.001$~0.02 by the Mann-Whitney U test, Fig 3A and Table 2). In the experiment of KIM-1 tumors, a significant reduction of tumor volume was also observed with the use of PEG-IFN-α2a ($P < 0.001$ by two-factor factorial ANOVA, Fig 3B). There were significant differences in the actual tumor weight between the Control group and the PEG-IFN-α2a groups, except for the PEG-IFN-α2a (0.06 μg) group (Table 2). The actual tumor weight at the end of the experiment #2 was summarized in Table 3. Subcutaneous injection of 0.6 μg of PEG-IFN-α2a induced the significant reduction of tumor weight, compared with the Control group and the group that received the same international unit of non-pegylated IFN-α2a ($P<0.005$ and $P<0.03$, respectively). In this experiment, there was no significant difference between the Control group and the PEG-IFN-α2a (0.06 μg) group ($P=0.078$).

Histological examination of the HAK-1B tumor specimens stained with HE revealed that the numbers of apoptotic cells in the mice treated with PEG-IFN-α2a (0.06 or 0.6 μg) were significantly higher than that of the Control, and the number increased dose dependently (Fig. 4, A and B; Table 4). The incidence of apoptosis in TUNEL-stained sections showed the same tendencies as those obtained in HE-stained sections (Fig. 4C and Table 4). Immunohistochemical examination of BrdU uptake in HAK-1B tumors revealed that there was no significant difference in BrdU labeling index between the Control and PEG-IFN-α2a (0.06 or 0.6 μg) groups (Table 4).

There was a significant difference in the number of blood vessels per unit area within the HAK-1B tumor between the Control mice and the mice that received 0.06 μg of PEG-IFN-α2a (Fig. 5; Table 5). The number of blood vessels in the borderline area between the tumor nodule and surrounding tissues tended to be

higher in the mice treated with PEG-IFN-α2a (0.06 or 0.6 μg) than the Control mice. There was no significant difference in the expression of bFGF and IL-8 in subcutaneous tumors between the PEG-IFN-α2a group and the Control group (Table 5).

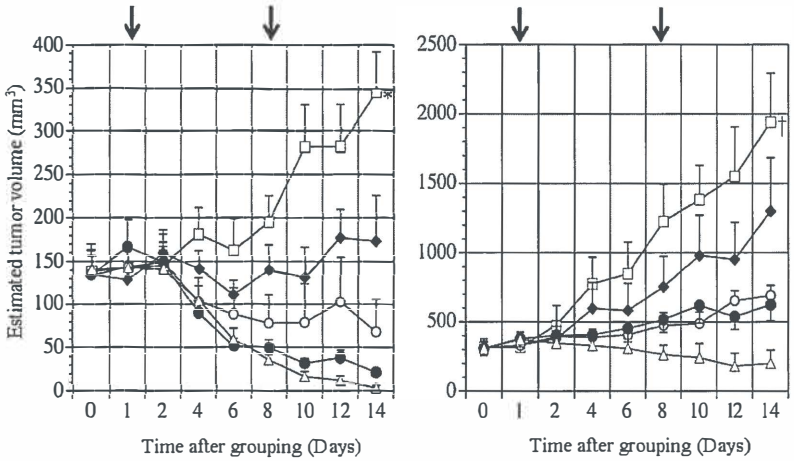

Figure 3. Time-course change in estimated tumor volumes of subcutaneously transplanted HAK-1B (A) or KIM-1 (B) tumors in nude mice in Experiment #1. The mice received a subcutaneous injection of 0.06 (♦), 0.6 (○), 6 (●), or 60 (△) μg of PEG-IFN-α2a, or medium alone (Control) (□), once a week for 2 consecutive weeks. The arrows show the days of injection. The figures represent average ± SE. *P < 0.0001, versus the other groups. †P < 0.01, versus the other groups.

Table 2. The weight of subcutaneous tumors of HAK-1B or KIM-1 cells in nude mice at killing Experiment #1

	Tumor weight (g)	
Treatment group[a]	HAK-1B	KIM-1
Control	0.303 ± 0.05[b, c]	1.050 ± 0.24[e]
PEG-IFN-α2a (0.06 μg)	0.141 ± 0.03[d]	0.725 ± 0.17[f]
PEG-IFN-α2a (0.6 μg)	0.033 ± 0.01	0.439 ± 0.04
PEG-IFN-α2a (6 μg)	0.015 ± 0.01	0.434 ± 0.04
PEG-IFN-α2a (60 μg)	0.0	0.076 ± 0.05

[a] Cultured HAK-1B or KIM-1 cells (1.0 X 10^7) were subcutaneously transplanted into nude mice. Mice in each group were treated with once per week sucutaneous injections of phosphate buffered saline (Control) or PEG-IFN-α2a. All mice were killed and the tumor weight was measured on the 15th day. [b] Mean ± SE. [c] P < 0.02, versus the PEG-IFN-α2a (0.06 μg) group; P < 0.001, versus the PEG-IFN-α2a (0.6 μg) group; P < 0.001, versus the PEG-IFN-α2a (6 μg) group. [d] P < 0.02, versus PEG-IFN-α2a (60 μg). [e] Not significant, versus the PEG-IFN-α2a (0.06 μg) group; P < 0.03, versus the PEG-IFN-α2a (0.6 μg) group; P < 0.05, versus the PEG-IFN-α2a (6 μg) group; P < 0.01, versus the PEG-IFN-α2a (60 μg) group. [f] P < 0.05, versus the PEG-IFN-α2a (60 μg) group.

Table 3. The weight of subcutaneous tumors of HAK-1B cell in nude mice at killing Experiment #2

Treatment group[a]	Tumor weight (g)
Control	0.726 ± 0.09[b, c]
IFN-α2a (0.0042 μg)	0.588 ± 0.07[d]
IFN-α2a (0.042 μg)	0.531 ± 0.04[e]
PEG-IFN-α2a (0.06 μg)	0.493 ± 0.04[f]
PEG-IFN-α2a (0.6 μg)	0.355 ± 0.03

[a] Cultured HAK-1B cells (1.0×10^7) were subcutaneously transplanted into nude mice. Mice in each group were treated with once per week sucutaneous injections of phosphate buffered saline (Control) or IFN-α2a or PEG-IFN-α2a. All mice were killed and the tumor weight was measured on the 15th day. [b] Mean ± SE. [c] $P < 0.005$, versus the PEG-IFN-α2a (0.6 μg) group. [d] $P < 0.02$, versus the PEG-IFN-α2a (0.6 μg) group. [e] $P < 0.03$, versus the PEG-IFN-α2a (0.6 μg) group. [f] $P < 0.02$, versus the PEG-IFN-α2a (0.6 μg) group

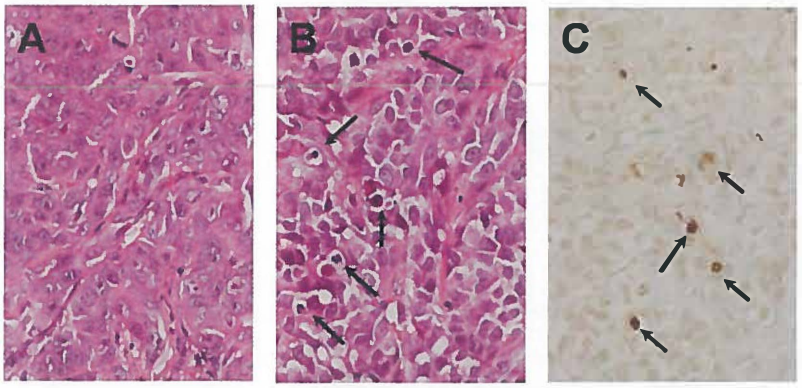

Figure 4. Photomicrograph of subcutaneous human HCC tumor in nude mice that was developed after the injection of HAK-1B cells. (A) A control mouse that received culture medium alone. The tumor shows a compact arrangement of tumor cells and a sinusoid-like structure in the stroma. (B) A mouse that received a s.c. injection of 0.06 μg of PEG-IFN-α2a. There are some apoptotic tumor-cells characterized by shrinkage and eosinophilic change in the cytoplasm, chromatin condensation and/or fragmentation of nuclei (arrows, HE staining, X200). (C) The same tumor as shown in (B). There are some TUNEL-positive cells showing brown nuclei (arrows, stained by the TUNEL technique, X200).

Table 4. Numbers of apoptotic cells and BrdU-positive cells in human HCC tumors subcutaneously transplanted in nude mice

	Apoptosis[b] (Number of cells/0.25mm^2)		BrdU Labeling Index[c] (Number of positive cells/0.25mm^2)
Treatment group[a]	HE stain	TUNEL method	
Control	8.4 ± 0.8[d, e]	9.6 ± 1.1[e]	32.3 ± 1.6[f]
PEG-IFN-α2a (0.06 μg)	12.2 ± 1.0	15.4 ± 1.8	27.0 ± 2.6
PEG-IFN-α2a (0.6 μg)	12.4 ± 0.9	16.1 ± 1.5	31.3 ± 6.9

[a] Cultured HAK-1B cells (1.0 X 10^7) were subcutaneously transplanted into nude mice. Mice in each group were treated with once per week subcutaneous injections of phosphate buffered saline (Control) or PEG-IFN-α2a. Tumors of mice that received 6 or 60 μg of PEG-IFN-α2a could not be used because the tumors were too small to evaluate. All mice were killed on the 15th day. [b] The number of apoptotic cells was counted in at least three 0.25 mm^2-areas in each section stained with hematoxylin and eosin, and the average number per area in each group was obtained. The number of TUNEL-positive cells was also counted in the same manner. [c] The number of BrdU-positive cells was counted in at least three 0.25 mm^2-areas in each section, and the average number per area in each group was obtained as the labeling index. [d] Mean ± SE. [e] $P < 0.02$, versus the other groups. [f] Not significant, versus the other groups.

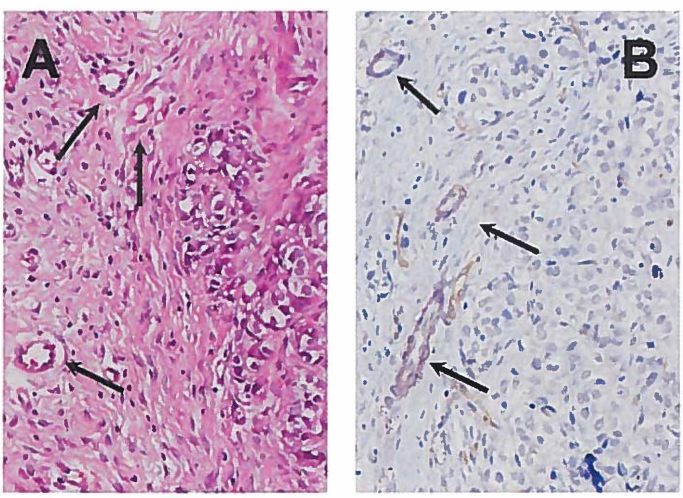

Figure 5. Photomicrograph showing double immunostained artery-like blood vessels in a subcutaneous human HCC tumor developed after the injection of HAK-1B cells. (A)
Photomicrograph of a subcutaneous HAK-1B tumor in a nude mouse that received a s.c. injection of 0.06 μg of PEG-IFN-α2a showing small artery-like blood vessels in the scar (arrows, HE staining, X200). (B) CD34/α-SMA double-positive artery-like blood vessels are noted (arrows, CD34/α-SMA double-immunostain, X200).

Table 5. Numbers of artery-like blood vessels, and Enzyme-linked immunosorbent assay (ELISA) of angiogenesis factors in human HCC tumors subcutaneously transplanted in nude mice

	Artery-like blood vessel[b] (Number of vessels/mm^2)			Levels in the tumor lysate[c] (pg/40 μg cellular protein)	
Treatment group[a]	Inside of the tumor	around the tumor	total number	bFGF	IL-8
Control	0.274 ± 0.09[d, e]	1.14 ± 0.25[f]	1.41 ± 0.33[g]	14.0 ± 1.8[f]	2.8 ± 1.0[f]
PEG-IFN-α2a (0.06 μg)	0.868 ± 0.16	1.30 ± 0.29	2.17 ± 0.43	19.8 ± 2.1	4.9 ± 1.3
PEG-IFN-α2a (0.6 μg)	1.87 ± 1.45	2.36 ± 0.88	4.23 ± 2.14		

[a] Cultured HAK-1B cells (1.0 X 10^7) were subcutaneously transplanted into nude mice. Mice in each group were treated with once per week subcutaneous injections of phosphate buffered saline (Control) or PEG-IFN-α2a. Tumors of mice that received 6 or 60 μg of PEG-IFN-α2a could not be used because the tumors were too small to evaluate. All mice were killed on the 15th day. [b] The number of blood vessels in and around the tumor nodule was counted on each section, and the average number per area in each group was obtained. [c] The expression levels of basic fibroblast growth factor (bFGF) and IL-8 of the resected tumors were measured by ELISA. [d] Mean ± SE. [e] $P < 0.002$, versus the PEG-IFN-α2a (0.06 μg) group; $P<0.05$, versus the PEG-IFN-α2a (0.6 μg) group. [f]. Not significant, versus the other groups. [g] $P < 0.05$, versus the PEG-IFN-α2a (0.6 μg) group.

Discussion

In the *in vitro* study, we showed that PEG-IFN-α2a inhibit the growth of 8 and 11 out of 13 cell lines in a time- and dose-dependent manner, however, PEG-IFN-α2a was apparently less active on an IC50 basis, compared with either PEG-IFN-α2b or IFN-α2b or consensus IFN-α or BALL-1 lymphoblastoid IFN-α which was tested in the same experimental condition in our previous reports [10, 18, 21]. For example, IC50 for HAK-1B cells was approximately 253 ng/ml of PEG-IFN-α2a, 13.1 ng/ml of PEG-IFN-α2b, 2.4 ng/ml of IFN-α2b, 0.7 ng/ml of consensus IFN-α and 1.1 ng/ml of BALL-1 lymphoblastoid IFN-α. On the other hand, in the *in vivo* study, s.c. injection of PEG-IFN-α2a once a week showed better antitumor effect on a tumor volume or weight basis, compared with that of non-pegylated IFN-α2a. These results might support our hypothesis that continuous contact with IFNs induces strong *in vivo* antitumor effects, and are not surprising because it was reported that PEG-IFN-α2a showed less active in vitro antiviral activity and but had much more in vivo antitumor activity than non-pegylated IFN-α2a [23]. Another interesting finding in the *in vitro* study is the discrepancy between the results of MTT assay and apoptosis detection assay. When HAK-1B or KIM-1 was cultured with PEG-IFN-α2a, IC50 for HAK-1B was much lower than that for KIM-1 although HAK-1B showed lower rate of apoptotic cells than KIM-1. These findings suggest that there might be some mechanisms other than apoptosis, which affect the sensitivity to antitumor effects of PEG-IFN-α2a. Factors related to the antitumor effect of IFNs

are still unclear. The expression of interferon receptor is one of the most studied factors. For instance, our previous report showed that IFN-α inhibited the proliferation of cultured HCC cells by inducing inhibition of cell-cycle progression with or without apoptosis, regardless of the expression level of type I IFN receptor beta chain [18]. On the other hand, Nagano et al reported that the expression of this type I IFN receptor on HCC tissue might be a useful predictor to find potential responder to INF-α/5-fluorouracil combination therapy [24]. Immunomodulation by IFNs has also been well studied as a factor related to antitumor effect. In this study, we used athymic mice, which lack mature T-cell, and human IFNs. Since IFNs are species-specific [25], we surmise that this immunomodulative effect is limited in our study, but this should be confirmed in the future study using mouse IFN.

Morphological observation of the subcutaneous tumors of nude mice revealed that s.c. injection of PEG-IFN-α2a induce the significant increase of apoptotic cells compared with Control group. This result in the *in vivo* study is consistent with that in the *in vitro* study showing characteristic changes of apoptosis after adding PEG-IFN-α2a. Although the inhibition of angiogenesis as well as the induction of apoptosis is regarded as one of the biological effects of IFNs, the number of artery-like blood vessels of the subcutaneous tumors in the treatment group was higher than that in the Control group. There are three possible explanations of this finding. Firstly, PEG-IFN-α2a was less effective for mouse endothelial cells compared with human cancer cells due to the species specificity of human IFNs. Secondly, these vessels are reflecting the increased angiogenesis in the granulation tissue surrounding tumor. Lastly but not least, it might be difficult to visualize the alteration in the number of vessels in order to examine the efficacy of drugs that possess antiangiogenic activity. Hlatky et al explained in their review article that the reason is that the tightness of the coupling between vessel drop-out and tumor-cell drop-out after the treatment is different [26]. We had observed similar findings in our previous report in which human HCC tumors subcutaneously transplanted in nude mice showed much apoptosis in either PEG-IFN-α2b or IFN-α2b treatment group compared with the Control group, but no significant difference in the number of blood vessels [10]. Kojiro et al also showed that s.c. injection of BALL-1 lymphoblastoid IFN-α increase the number of artery-like blood vessels and the protein expression of bFGF within HCC xenograft tumors in spite of the significant decrease of actual tumor weight [27]. In contrast, Dinney et al showed that IFN-α2a decreases the blood vessel density and the expression of bFGF in orthotopic xenograft model of bladder tumor [28]. The reason for these contrary findings remains unclear and further evaluation with caution is needed by using different doses and types of IFNs and different cell lines.

The association between IFN therapy and occurrence or recurrence of HCC has been investigated in some reports. HALT-C trial group showed in their randomized

control trial in a large cohort that long-term PEG-IFN-α2a therapy does not reduce the incidence of HCC among patients with chronic HCV infection who have previously failed to achieve a sustained virologic response to therapy [29]. Among only patients with cirrhosis, long-term PEG-IFN-α2a therapy reduced a risk of HCC after a long-time observation [30]. EPIC study group also showed long-term PEG-IFN-α2b therapy does not prevent HCC [31]. On the other hand, Nishiguchi et al reported that long-term IFN-α therapy after curative resection of HCV-related HCC prolongs the survival rate, although preventive effect of intrahepatic recurrence was marginal [32]. Sakaguchi et al also showed that among patients who underwent radical radiofrequency therapy for HCV-related HCC, long-term IFN-α2b therapy reduced the recurrent rate of HCC [4]. These reports with conflicting results may be suggesting that IFN therapy is effective only after the initial curative treatment of HCV-related HCC. In addition, there are several reports that support that IFN therapy prevents the development of HCC among patients with chronic HBV infection or those underwent curative resection of HBV-related HCC [5, 7].

In conclusion, we demonstrated antitumor effect of PEG-IFN-α2a for human liver cancer cells *in vitro* and *in vivo* and our results suggest that longer contact to IFNs may induce stronger antitumor effect in body. PEG-IFN-α2a might be a possible treatment option for HCC as well as chronic viral hepatitis. Further studies are needed from both molecular and clinical view points in order to find out particular patient group those respond to this therapy.

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Summary

Hepatocellular carcinoma (HCC) is the sixth most common cancer globally, and the third most common cause of cancer-related death. Most HCCs occur in a cirrhotic liver which develops as a consequence of longstanding inflammation, and this fact is an important factor that contributes to the limitations of therapeutic management of HCC. HCC treatment strategy is based not only on tumor status, e.g. size and the number of tumor, but also on the patients' residual liver function. According to the Barcelona Clinic Liver Cancer staging system, which is the most widely applied system, HCC patients at the very early or early stage can be candidates for potentially curative treatment options, such as liver transplantation, surgical resection, and radiofrequency ablation. One of the most important negative prognostic factors of surgical treatment of HCC is the presence of microvascular invasion (micro-VI). Micro-VI is a frequent phenomenon for which there is no reliable preoperative predictive factor. Patients with advanced stage HCC, who unfortunately represent the major group of HCC patients at the time of diagnosis, have the limited option of palliative treatment. Sorafenib, an oral multikinase inhibitor, is the first and so far the only single systemic agent which can improve overall survival of patients with advanced HCC by several months.

This thesis addresses two issues that influence the prognosis of HCC, micro-VI (Part I) and Interferon (IFN) therapy (Part II).

Part I: The significance of Microvascular invasion

Micro-VI is currently regarded as an independent prognostic factor, and can only be identified by postoperative histopathological assessment due to its microscopic nature. In **chapter 2** we reviewed the current status of methods to predict micro-VI preoperatively and the prognostic value of micro-VI in patients who underwent liver transplantation for HCC. Many studies reported the significant correlation between micro-VI and size and number of tumors. The likelihood of micro-VI can be predicted on the basis of tumor size and number preoperatively, however an important limitation is the difficulty of accurate radiological assessment of tumor size and number in a cirrhotic liver. The association of the gross appearance of HCC

with micro-VI has been reported repeatedly mainly by Japanese studies. However, a limitation is that it is still not possible to evaluate the gross type by conventional imaging modalities. New imaging modalities and serum biomarkers were also reviewed as a potential tool to predict micro-VI before surgery. It was noted that fluorodeoxyglucose positron emission tomography and the serum concentration of des-γ-carboxy prothrombin can be predictive although further validation studies are needed. We concluded that micro-VI is an independent risk factor for recurrence and survival after liver transplantation and that currently there is no reliable predictive marker for micro-VI before surgery.

Despite the significance of micro-VI, its molecular background remains obscure. Vascular invasion requires several processes at sites where the primary tumor develops: tumor cell proliferation; epithelial-mesenchymal transition (EMT); extracellular matrix degradation, cell migration and tumor angiogenesis. To establish a potential profile of micro-VI positive HCC, in **chapter 3**, we investigated micro-VI in HCCs from the perspective of a 3-step process; tumor characteristics, angiogenesis and EMT. We studied the gene expression profile of factors associated with these processes. High grade tumor, decreased E-cadherin expression on tumor cells, and an increased placental growth factor expression in non-cancerous peritumoral tissue were associated with the presence of micro-VI. In addition, in the micro-VI positive group the expression of placental growth factor and vascular endothelial growth factor receptor-1 was increased in peritumoral tissue compared with the corresponding HCC. The altered angiogenic status in peritumoral tissue highlighted the significance of the microenvironment in which micro-VI develops and the possible crosstalk between cancer cells and tumor stroma.

To further investigate the changes in tumor stroma associated with micro-VI, in **chapter 4** we focused on the changes in the peritumoral fibrous septa which were collected by laser microdissection. We studied the expression pattern of the same set of genes as in chapter 3. Our results showed that in the micro-VI positive group there was a decreased gene expression of epithelial cell adhesion molecule (EpCAM) and E-cadherin within peritumoral septa compared with the expression in cirrhotic septa collected distant from the tumor. Septal EpCAM and E-cadherin

expression could only be found in biliary ductules since these were the only epithelial cell component present in the septa. Subsequent comparative quantification of septal ductules revealed that micro-VI positive HCC was associated with a significantly lower numbers of peritumoral ductules compared to the number of ductules in the corresponding distant septa. This finding may represent a potential tool to predict micro-VI in biopsies before surgery provided robust validation studies can confirm the association that we found.

Part II: Systemic therapy for HCC: the role of interferon therapy

It is well known that IFNs possess antitumor effect against certain types of cancer as well as antiviral activity. Pegylated IFN alpha (PEG-IFN-α) which has longer serum half-life in body than the conventional one is used to treat patients with hepatitis C virus infection in combination with a nucleoside analog, ribavirin as a standard care. In addition, it was reported that IFNα can prolong patient's survival in inoperable HCC. Combination therapy of PEG-IFN-α with sorafenib may have a potential to improve the outcome of sorafenib monotherapy. In **chapter 5**, we investigated the antiproliferative effects of combination therapy of sorafenib and PEG- IFN-α2b on two human HCC cell-lines *in vitro* and *in vivo*. In the *in vitro* study, a synergistic or additive antiproliferative effect of combination therapy was seen. In the *in vivo* study, a significant reduction of tumor volume and weight was observed in the combination group, although the effect of the monotherapy group was not always significant. In **chapter 6**, we investigated the effects of PEG-IFN-α2a on the growth of human liver cancer cells. PEG-IFN-α2a inhibited the growth *in vitro*, and its 50% growth inhibitory concentrations were higher than non-pegylated IFN-α2a. On the other hand, PEG-IFN-α2a showed better antiproliferative effect in the *in vivo* study than non-pegylated one possibly due to their unique pharmacokinetics. These findings in **chapter 5 and 6** suggest that PEG-IFNs might be potential agents for the treatment of HCC with or without sorafenib. The different sensitivities of various HCC cell-lines to IFN or sorafenib therapy also suggest that molecular profiling of the tumor will be necessary for a better matching of tumor with several systemic drugs.

Nederlandse Samenvatting

Hepatocellulair carcinoom (HCC) staat wereldwijd op de zesde plaats van de meest voorkomende kanker soort en op de derde plaats van de meest voorkomende kanker gerelateerde doodsoorzaak. De meeste HCCs komen voor in cirrotische levers die het gevolg zijn van een langdurig ontstekingsproces en dit is een belangrijke factor die bijdraagt aan de beperkingen van de therapeutische mogelijkheden voor HCC. Het behandelbeleid van HCC is niet alleen gebaseerd op de tumorstatus, zoals grootte en aantallen tumor nodi, maar ook op de status van de leverfuncties van de patiënt. Volgens het meest toegepaste *Barcelona Clinic Liver Cancer* stageringsschema zouden HCC patiënten die zich in het zeer vroege stadium bevinden of in het vroege stadium, kandidaten zijn voor een potentieel curatieve therapeutische optie zoals lever transplantatie (LT), chirurgische resectie en *radio frequency ablation*. Een van de belangrijkste negatieve prognostische factoren van de chirurgische therapie van HCC is microvasculaire invasie (micro-VI). Micro-VI is een frequent fenomeen waarvoor geen betrouwbare preoperatieve voorspellende methode bestaat. Patiënten met een gevorderd stadium van HCC, die helaas het grootste deel van de HCC patiënten vertegenwoordigen ten tijde van de diagnose, hebben een beperkte optie van palliatieve behandeling. Sorafenib, een orale multikinase remmer is het eerste en tot dusver enige systemische middel die tot een verbetering van de *overall* overleving van enkele maanden kan leiden bij HCC patiënten in een gevorderd stadium.
In dit proefschrift behandelen we 2 onderwerpen die invloed hebben op de prognose van HCC, namelijk micro-VI (deel I) en Interferon (IFN) therapie (deel II).

Deel I: De rol van Microvasculaire Invasie
Micro-VI wordt tegenwoordig beschouwd als een onafhankelijk prognostische factor maar kan vanwege de microscopische aard ervan pas postoperatief worden vastgesteld bij histologisch onderzoek van de gereseceerde tumor. **Hoofdstuk 2** is een overzicht waarin we de huidige stand van zaken betreffende de preoperatieve opsporingsmethoden van micro-VI hebben geëvalueerd evenals de prognostische waarde van micro-VI bij patiënten met HCC die een LT hebben ondergaan. In veel studies werd een significante correlatie gezien tussen micro-VI en de grootte van de tumor en het aantal tumornodi. De mogelijke aanwezigheid van micro-VI kan preoperatief worden voorspeld op basis van de grootte en het aantal tumoren maar een nauwkeurige radiologische evaluatie van deze twee eigenschappen is in een cirrotische lever bijzonder moeilijk. In veel Japanse studies werd het verband gelegd tussen het macroscopisch aspect van de tumor met micro-VI maar de beperkende factor is het feit dat het nog steeds niet mogelijk is om het

macroscopisch aspect van de tumor preoperatief vast te stellen met de huidige *imaging* technieken. Bij de evaluatie van nieuwe *imaging* technieken en serum biomarkers kwamen *fluorodeoxyglucose positron emission tomography* en serum gehalte van *des-γ-carboxy prothrombin* naar voren als potentiële mogelijkheden voor preoperatieve vaststelling van micro-VI. Verdere validatie studies zijn echter noodzakelijk. We kwamen tot de conclusie dat micro-VI een onafhankelijke risicofactor is voor HCC recurrens na LT die de survival negatief beïnvloedt en waarvoor tegenwoordig nog geen betrouwbare middel bestaat voor de preoperatieve opsporing ervan.
Ondanks het feit dat micro-VI een belangrijk fenomeen is, is er weinig bekend over de moleculaire achtergrond ervan. Vasculaire invasie omvat meerdere processen op de plaats waar de primaire tumor zich ontwikkelt: tumorcel proliferatie, epitheliale-mesenchymale transitie (EMT), extracellulaire matrix degeneratie, celmigratie en tumorangiogenese. In **hoofdstuk 3** hebben we getracht een moleculair profiel op te stellen van micro-VI positieve HCC. Hiervoor onderzochten we micro-VI in HCC vanuit het perspectief van een 3-staps proces dat de tumor eigenschappen, EMT en angiogenese omvat. We onderzochten het genexpressieprofiel dat geassocieerd is met deze 3 processen. Het voorkomen van micro-VI bleek te zijn geassocieerd met een hoge tumorgraad, verlaagde E-cadherin expressie in tumorcellen en een toegenomen expressie van *placental growth factor* in het leverweefsel naast de tumor. Daarnaast werd in de micro-VI positieve HCC groep een hogere expressie gezien van *placental growth factor* en *vascular endothelial growth factor receptor-1* in het peritumorale leverweefsel ten opzichte van de daarnaast gelegen HCC. Van *placental growth factor* is bekend dat het pathologische angiogenese stimuleert. De veranderde angiogene status in het peritumorale leverweefsel benadrukt de belangrijke rol van de micro-omgeving waarin micro-VI plaats vindt en de *crosstalk* tussen kankercellen en tumorstroma.

Verder onderzoek naar veranderingen in het tumorstroma werd verricht in **hoofdstuk 4.** In dit onderzoek werd peritumoraal fibreus stroma geïsoleerd middels lasermicrodissectie en hierin werd het expressiepatroon van dezelfde groep genen als in hoofdstuk 3 onderzocht. In het peritumorale stroma van de micro-VI positieve HCC groep werd een verlaagde expressie gezien van *epithelial cell adesion molecule* (EpCAM) en E-cadherin in vergelijking met de expressie in het fibreuze stroma afkomstig van cirrhotische septa die verder van de tumor gelegen waren. EpCAM en E-cadherin kunnen in fibreuze septa uitsluitend tot expressie komen in biliaire ductuli omdat deze de enige epitheliale cellen herbergen die in dit compartiment aanwezig kunnen zijn. De kwantitatieve vervolgstudie toonde aan dat micro-VI positieve HCC geassocieerd was met een significant lager aantal ductuli in het peritumorale stroma ten opzichte van het verder gelegen

cirrhotische stroma. Deze bevinding zou een mogelijkheid kunnen bieden om micro-VI in biopten preoperatief te kunnen voorspellen als de resultaten goed zijn gevalideerd.

Deel II: systemische therapie van HCC: de rol van interferon therapie

Het is bekend dat interferon (IFN) zowel een antitumor effect heeft ten opzichte van een aantal kankersoorten als een antivirale activiteit. Gepegyleerd IFN alpha (Peg-IFN-α) dat een langere halfwaarde tijd heeft in het lichaam dan de conventionele types wordt in combinatie met een nucleoside analoog, ribavirin, gebruikt als standaard behandeling van hepatitis C. Daarnaast werd IFN-α genoemd als een middel dat de overleving kan verlengen van patiënten met een inoperabele HCC. Op grond hiervan kwamen we tot de hypothese dat een combinatie therapie van Sorafenib met Peg-IFN-α het effect van Sorafenib monotherapie zou kunnen verbeteren. In **hoofdstuk 5** werd het antiproliferatie effect van de combinatie van Sorafenib en Peg-IFN-α2b onderzocht op 2 humane HCC cellijnen zowel *in vitro* als *in vivo*. In de *in vitro* studie werd een synergistisch of additief effect van de combinatie therapie gezien. In het *in vivo* deel werd een significante reductie gezien van het volume en gewicht van de tumor in de combinatie-therapie groep, maar er was geen synergistisch effect. In **hoofdstuk 6** werd het effect onderzocht van Peg-IFN-α2a op de groei van humane HCC cellijnen. Peg-IFN-α2a remde de groei *in vitro*, en de 50% groeiremmende concentraties waren hoger dan ongepegyleerd IFN-α2a. Maar ongepegyleerd Peg-IFN-α2a toonde wel een beter antiproliferatie effect in het *in vivo* model dan IFN-α2a, vermoedelijk door de verschillen in hun farmacokinetiek. De bevindingen van de **hoofdstukken 5 en 6** suggereren dat PEG-IFN potentiële middelen zouden kunnen zijn voor de behandeling van HCC met of zonder Sorafenib. De verschillende gevoeligheden van de verschillende HCC cellijnen ten opzichte van IFN en Sorafenib therapie suggereren ook dat moleculaire *profiling* van de tumor noodzakelijk zal zijn voor een betere afstemming tussen de tumor en de verschillende typen systemische geneesmiddelen.

日本語要旨

肝細胞癌は、肝原発悪性腫瘍の約 9 割を占め、世界における全ての癌の中で発生率 6 位、癌関連死の 3 位に位置している。また多くの肝細胞癌は、長期にわたる肝の炎症の終末像である肝硬変を背景として発生し、そのことが治療を制約する要因となっている。肝癌の治療方法の決定においては、腫瘍の大きさや数のみならず、患者の肝予備能も考慮せねばならない。このように腫瘍因子と肝予備能を統合したステージングシステムとして世界で広く利用されているものに Barcelona Clinic Liver Cancer (BCLC)ステージングシステムがある。BCLC ステージングシステムでは超早期および早期の肝細胞癌の患者は肝移植や肝切除術、ラジオ波焼灼術など治癒を目的とした治療の対象となる。しかしながら多くの患者は進行期に発見され、その治療法は対症療法など限られたもののみである。経口マルチキナーゼ阻害剤であるソラフェニブは、進行期の肝細胞癌の予後を改善することが証明された世界初の、そして現時点において唯一の、薬物である。この学位論文では、微小血管侵襲とインターフェロン療法という 2 つの肝細胞癌の予後に関わるものを研究の対象とした。

第 1 部：微小血管侵襲の重要性について

微小血管侵襲は、肝細胞癌における独立した予後規定因子とみなされているが、その定義ゆえに術後の摘出標本の病理組織学的検索によってのみしか発見し得ない。第 2 章では、微小血管侵襲の術前予測の試みの現況について概説した。多くの研究が、腫瘍の大きさや数と微小血管侵襲の有無に関連があることを報告している。しかしながら術前に、硬変肝に発生した肝細胞癌の大きさと数を正確に評価することは依然として困難である。また肝細胞癌の肉眼型が微小血管侵襲の有無とよく相関することが主として日本から報告されているが、同様に術前画像での予測が困難である。その他に FDG-PET などの新しいモダリティーや PIVKA-II などの血清マーカーによる術前予測の試みについても検討したが、いずれもまだ十分な検証がなされていない。現段階においては微小血管侵襲を術前に予測するための信頼するに足る方法は無い、というのが我々の結論である。

微小血管侵襲の分子生物学的なメカニズムは依然として完全には解明されていない。一般的に血管侵襲は、原発巣での腫瘍の増殖、上皮-間葉転換(EMT)、細胞外マトリクスの破壊、腫瘍細胞の浸潤、腫瘍血管新生といった過程を経て完成すると考えられている。第 3 章において我々は、腫瘍の特性・EMT・血管新生という 3 つの点に着目して、これらに関与する遺伝子の発現が微小血管侵襲の有無によってどのように変化するかを検討したところ、低分化肝細胞癌、腫瘍部における E-カドヘリン発現の低下、周辺の非腫瘍部組織における胎盤増殖因子(PlGF)の発現の増加が微小血管侵襲の存在と関連していることがわかった。また、微小血管侵襲を有する肝細胞癌においてのみ、腫瘍部より周辺の非腫瘍部組織における PlGF およびその受容体である血管内皮増殖因子受容体(VEGFR)-1 の発現の増加が見られることがわかった。これらの周辺非腫瘍部における血管新生因子およびその受容体の遺伝子発現の変化は、腫瘍細胞とその微小環境における非腫瘍細胞との間に何らかの相互作用が存在していることを示唆するものと考えられた。第 4 章では非腫瘍部組織に着目し、腫瘍周囲および腫瘍から 3cm 以上離れた部位の線維性隔壁のみをレーザーマイクロダイセクションにて採取し、同部位における遺伝子発現の変化について検討した。微小血管侵襲の有無による遺伝子発現レベルの違いは、腫瘍周辺部・腫瘍から 3cm 以上離れた部位いずれにおいても認められなかった。しかしながら、腫瘍周辺部より腫瘍から 3cm 以上離れた部位の方が EpCAM および E-カドヘリンの発現が高いという結果が、微小血管侵襲を有する肝細胞癌においてのみ認められた。対象組織である線維性隔壁内において EpCAM もしくは E-カドヘリン陽性細胞は細胆管のみであるため、EpCAM の免疫染色を行い、EpCAM 陽性細胆管の数の比較検討を同じ症例を用いて行った。この検討では有意差は得られなかったものの、対象症例数を増やした新規の症例群では、遺伝子発現解析の結果と同様に、微小血管侵襲を有する群においてのみ、EpCAM 陽性細胆管の数が腫瘍周辺部より腫瘍から 3cm 以上離れた部位の方が多いという結果が得られた。生検組織を用いた検証が必要だが、術前に微小血管侵襲の有無を予測するためのツールになり得るものと考えられた。

第 2 部：肝細胞癌の全身療法: インターフェロン療法の役割について

インターフェロン(IFN)は抗腫瘍効果・抗ウイルス効果を有することが知られており、過去には切除不能肝細胞癌の予後を改善するという報告もなされている。また従来の IFN とくらべより長い血中半減期を有するペグインターフェロン-α(PEG-IFN-α)は、核酸アナログであるリバビリンとの併用することで、慢性 C 型肝炎の標準治療薬となっている。我々は PEG-IFN-α をソラフェニブと併用することで、肝細胞癌に対してより高い治療効果を示すのではないかと考え、PEG-IFN-α の in vivo および in vitro での肝細胞癌に対する抗腫瘍効果について検討した。第 5 章では PEG-IFN-α2b とソラフェニブの併用効果について 2 種類の肝細胞癌株を用いて検討した。in vitro では併用により PEG-IFN-α2b もしくはソラフェニブ単独療法とくらべ相乗的もしくは相加的な効果が得られた。ヌードマウスの皮下に細胞株を移植した in vivo の実験では、単独療法群では腫瘍の体積・重量の減少が必ずしも有意ではなかったが、併用療法群では有意な減少が認められた。第 6 章では PEG-IFN-α2a の肝癌細胞株に対する抗腫瘍効果を、従来の非ペグ化 IFN-α2a と比較検討した。in vitro の実験で得られた 50%阻害濃度は、PEG-IFN-α2a の方が非ペグ化製剤より高かったが、in vivo の実験では PEG-IFN-α2a の方がより高い抗腫瘍効果を示した。この in vitro と in vivo での相反する結果は、ペグ化製剤の薬物動態を考慮すると、妥当なものと考えられた。第 5, 6 章で得られた結果は、PEG-IFN-α が肝細胞癌に対する有用な治療法になり得る可能性を示すものだが、一方で肝細胞癌株によって感受性が大きく異なっていたことから、より治療に効果を示す患者群を特定するための検討が必要と考えられた。

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